Receptor-mediated Transcytosis of IgA in MDCK Cells Is via Apical Recycling Endosomes

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Abstract. Classically, the polymeric immunoglobulin receptor and its ligand, IgA, are thought to be sorted from basolateral early endosomes into transcytotic vesicles that directly fuse with the apical plasma membrane. In contrast, we have found that in MDCK cells IgA is delivered from basolateral endosomes to apical endosomes and only then to the apical cell surface. When internalized from the basolateral surface of MDCK cells IgA is found to accumulate under the apical plasma membrane in a compartment that is accessible to two apically added membrane markers: anti-secretory component Fab fragments, and avidin internalized from the biotinylated apical pole of the cell. This accumulation occurs in the presence of apical trypsin, which prevents internalization of the ligand from the apical cell surface. Using a modification of the diaminobenzidine density-shift assay, we estimate that approximately 80% of basolaterally internalized IgA resides in the apical endosomal compartment. In addition, approximately 50% of basolaterally internalized transferrin, a basolateral recycling protein, has access to this apical endosomal compartment and is efficiently recycled back to the basolateral surface. Microtubules are required for the organization of the apical endosomal compartment and it is dispersed in nocodazole-treated cells. Moreover, this compartment is largely inaccessible to fluid-phase markers added to either pole of the cell, and therefore seems analogous to the recycling endosome described in nonpolarized cells. We propose a model in which transcytosis is not a specialized pathway that uses unique transcytotic vesicles, but rather combines portions of pathways used by non-transcytosing molecules.

A primary function of epithelial cells is to regulate the exchange of macromolecules between their external environment and the underlying tissue. This selective exchange is made possible by the impermeability of the monolayer to both large and small molecules and specialized endocytotic pathways that allow transit of macromolecules across these cells (Mostov et al., 1992). Epithelia characteristically have discrete plasma membrane domains at the apical and basolateral poles of the cell, that are separated by tight junctions (Simons and Wandinger-Ness, 1990). Each domain has a distinct protein and lipid composition which is maintained despite an enormous flux of membrane traffic to and from each surface. In MDCK cells, for instance, an area of plasma membrane equivalent to 40% of the cell surface is endocytosed per hour (von Bonsdorff et al., 1985). Fluid-phase material endocytosed from the apical and basolateral surfaces of these cells is delivered to correspondingly distinct apical and basolateral early endosomes that are not thought to interchange their contents (Bomsel et al., 1989; Parton et al., 1989). Although much of the endocytosed fluid, and many membrane components are recycled to the original plasma membrane surface, some are sent in a microtubule dependent process to a common late endosome or prelysosome, where material endocytosed from the two surfaces meets (Bomsel et al., 1989; Parton et al., 1989; Bomsel et al., 1990; Fujita et al., 1990). In addition, some proteins are sorted in early endosomes into transcytotic vesicles and delivered to the opposite plasma membrane surface.

Transcytosis is a key membrane trafficking process in polarized cells because it allows for the exchange of macromolecules from one cell surface to the opposite one (Apodaca et al., 1991; Mostov et al., 1992). In addition, this is the only pathway for delivery of newly synthesized membrane proteins to the apical cell surface that is universally found in all epithelial cells examined, and in some epithelial cells, such as hepatocytes, it is virtually the only pathway for membrane proteins to this surface (Bartles et al., 1987). Transcytosis is also central to the establishment of cell polarity (Zurzolo et al., 1992).

Much of our understanding of the transcytotic pathway comes from studies of the polymeric immunoglobulin receptor (pIgR)[1] and its ligand dimeric IgA. The generally accepted model of pIgR traffic (Fig. 1) is based on studies in rat liver and MDCK cells transfected with the pIgR cDNA (Geuze et al., 1984; Hoppe et al., 1985; Apodaca et al., 1991). The pIgR and its ligand are internalized, along with the other receptors and fluid phase markers, in coated pits

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1. *Abbreviations used in this paper*: DAB, diaminobenzidine; FSG, fish skin gelatin; MTOC, microtubule organizing center; pIgR, polymeric-immunoglobulin receptor; SC, secretory component, Tf, transferrin.

at the basolateral (sinusoidal) surface (step 1). Subsequently, the receptor is delivered to a tubulo-vesicular early endosomal compartment shared with other receptors. Here the pIgR and its ligand are segregated from recycling receptors into microdomains of endosomal tubules and packaged into structures, which at least in thin sections have the appearance of vesicles (step 2) (Geuze et al., 1984). These "transcytotic vesicles" are thought to contain a variety of transcytosing molecules (Barr and Hubbard, 1993), but not recycling molecules (Sztul et al., 1991). In both hepatocytes and pIgR-expressing MDCK cells transcytotic vesicles are found accumulated near and in continuity with the apical (bile canalicular) cell surface (Geuze et al., 1984; Hoppe et al., 1985; Hunziker et al., 1990). Microtubules are required for this accumulation of transcytotic vesicles to occur. In nocodazole-treated MDCK cells IgA is internalized into basolateral endosomes but translocation of IgA from the basolateral to apical pole of the cells is prevented (Hunziker et al., 1990). Upon reaching the apical surface, the pIgR is cleaved to SC, which is released with IgA into secretions (step 3). A vesicular fraction highly enriched in pIgR and IgA has been isolated from rat liver and termed transcytotic carrier vesicles (Sztul et al., 1991). These vesicles have been shown to fuse with isolated bile canalicular membranes in a cell-free fusion assay that reconstitutes the fusion of transcytotic vesicles with the apical surface (Sztul et al., 1993).

We were interested in investigating the nature of the subapical IgA-containing vesicles/tubules found in hepatocytes and in pIgR-expressing MDCK cells. Do they represent transcytotic vesicles that are waiting to fuse with the apical surface, as has been suggested by several investigators (Geuze et al., 1984; Hoppe et al., 1985; Hunziker et al., 1990)? Alternatively does the apical accumulation of IgA represent ligand that has been internalized from the apical cell surface into apical endosomes, or IgA delivered from basolateral endosomes directly to apical endosomes? In addition, we wished to determine at what point in the transcytotic pathway IgA is segregated away from macromolecules that are either delivered to prelysosomes, (e.g., fluid-phase markers), or recycled back to the basolateral cell surface (e.g., transferrin). To address these questions we have analyzed the transcytotic pathway of IgA in pIgR-expressing MDCK cells. Using these cells it is possible to follow the distribution of IgA in relationship to ligands and fluid-phase markers that define the major endocytotic pathways, and to analyze events occurring at both the apical and basolateral poles of the cells. In contrast to the model for transcytosis described above, we find that in MDCK cells IgA is delivered from basolateral endosomes to apical endosomes and only then to the apical cell surface. In addition, we find that transferrin (Tf), a basolateral recycling protein, has access to this apical endosomal compartment, where it is recycled back to the basolateral surface.

Materials and Methods

Antibodies and Proteins

Purified human dimeric IgA was kindly provided by J.-P. Vaerman (Catholic University of Louvain, Brussels, Belgium) and was used at a concentration of 50 μg/ml. IgA-HRP was custom prepared from human dimeric IgA and HRP by Zymed (South San Francisco, CA) and used at 25 μg/ml. Rat monoclonal antibody ascites to ZO-1, a protein associated with tight junctions, was obtained from Chemicon (Temecula, CA) and was used at 1:100 dilution. Guinea pig anti-SC Fab fragments (derived from affinity purified guinea pig serum) were used at 10–20 μg/ml and were prepared as described (Breitfeld et al., 1989*b*). Fab-HRP was prepared by conjugating reduced Fab' to sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce Chemical Co., Piscataway, NJ) derivatized HRP according to the manufacturer's protocol and was used at 25 μg/ml. Avidin-TRITC and avidin-HRP were obtained from Vector Laboratories (Burlingame, CA) and used at 25 μg/ml. ExtrAvidin-10 nm gold was from Sigma (St. Louis, MO) and was washed with PBS and diluted 1:5. Lysine-fixable dextran-FITC (10,000 mol wt) was from Molecular Probes (Eugene, OR) and used at 10–15 mg/ml. Rabbit antiserum to the *trans*-Golgi network resident protein TGN-38 (kindly provided by G. Banting, University of Bristol, Bristol, England) has been described (Wilde et al., 1992) and was used at 1:100 dilution. Canine apo-transferrin (Sigma) was loaded with iron as described (Podbilewicz and Mellman, 1990) and used at 25 μg/ml. In addition this protein was coupled to keyhole limpet hemocyanin and the conjugate used to produce rabbit antibodies against this protein (coupling and immunization of rabbits was performed by Caltag, South San Francisco, CA). Anti-Tf antibodies were affinity purified on a canine-Tf sepharose column and used at 10–20 μg/ml. The mouse monoclonal antibody, H68.4, was kindly provided by I. Trowbridge (Scripps Research Institute, La Jolla, CA) and recognizes the Tf receptor from a number of different species (White et al., 1992). Expression of the Tf receptor in pIgR-expressing MDCK was confirmed by immunoprecipitating the 95,000-M_r protein from radiolabeled cell lysates. For immunofluorescence studies the ascites was diluted 1:100. AC17 is a mouse monoclonal antibody (kindly provided by E. Rodriguez-Boulain, Cornell University Medical College, New York, NY) to a 95,000-M_r lysosomal membrane glycoprotein and was used at 1:100 dilution (Nabi et al., 1991). The mouse monoclonal antibody DM5α, specific for α-tubulin, was obtained from Sigma and used at 1:250 dilution. A rabbit antiserum specific for γ-tubulin (kindly provided by T. Stearns, University of California, San Francisco, CA) has been described (Stearns et al., 1991) and was used at a 1:100 dilution. FITC- or Texas red–conjugated goat anti-human IgA, goat anti-rat IgG, goat anti-mouse IgG, goat anti-rabbit IgG antibodies were obtained from Jackson ImmunoResearch Laboratories (WestGrove, PA) and used at 10–20 μg/ml. The anti-rat, anti-mouse, and anti-rabbit antibodies had minimal cross-reactivity with each other and with human serum proteins.

Cell Culture

MDCK strain II cells expressing the wild-type rabbit pIgR have been described (Breitfeld et al., 1989*a*). Although the results presented in this paper are from one clone of MDCK strain II cells transfected with the pIgR cDNA, we have obtained very similar results using two other independent clones of pIgR-expressing MDCK cells (not shown). Cells were maintained in Minimal Essential Medium (MEM; obtained from the UCSF Cell Culture Facility) supplemented with 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2/95% air. In order to maintain a high level of receptor expression, new cells were thawed every 3–4 wk, and were split 1:10 and passaged once weekly. For all experiments, cells were cultured on 12-mm diam, 0.4-μm pore size Transwells (Costar; Cambridge, MA) as described (Breitfeld et al., 1989*a*). The cells were fed everyday and used 3–4-d postculture.

Internalization of Ligands and Fluid-phase Markers, Nocodazole Treatment, Stripping of Cell-surface Ligands, and Biotinylation

Ligands and fluid phase markers were internalized from the apical and/or basolateral surface of filter-grown MDCK cells. Prior to Tf internalization, it was necessary to incubate the cells for a minimum of 4 h at 37°C in MEM/BSA (MEM, Hank's Balanced Salts, 0.6% wt/vol BSA, 20 mM Hepes, pH 7.4) to deplete intracellular stores of Tf. Otherwise, a Tf-dependent signal could not be obtained in our immunofluorescence analysis. All incubations in MEM/BSA were performed in a circulating water bath. It was also possible to deplete the cells of Tf by incubating them overnight in MEM, Earle's balanced salts, 0.6% BSA, 20 mM Hepes, pH 7.4 in the tissue culture incubator. No obvious harmful effects were observed in serum-starved cells incubated in either fashion, as the distribution of pIgR and Tf-receptor were unaltered and the appropriate ligands were faithfully recycled or transcytosed. For basolateral uptake of ligands or fluid-phase markers the cells were rinsed with MEM/BSA at either 18° or 37°C

and the edge of the filter on the side opposite the cells was carefully blotted to remove excess medium. The Transwell unit was placed on a 25-μl drop of MEM/BSA containing the ligand or fluid-phase marker. For apical uptake, the cells were rinsed with MEM/BSA at the appropriate temperature, excess fluid was aspirated from the cell-side of the Transwell and 150 μl of ligand or fluid-phase marker, diluted in MEM/BSA, was added. All incubations were performed in a humid chamber. At the end of the experiment cells were typically washed two to three times with MEM/BSA equilibrated at the appropriate temperature, and either rapidly cooled down to 4°C or if appropriate fixed immediately.

Nocodazole (Calbiochem-Behring Corp., San Diego, CA) was dissolved in DMSO at 33 mM and stored at −20°C. In all experiments in which this drug was used, cells were pretreated 60 min at 4°C in the presence of 33 μM nocodazole. The drug was included in subsequent incubations.

In many of the experiments cell-surface receptors and their ligands were stripped from the cell surface as follows: cells were treated for 30 min at 4°C with 50–100 μg/ml of TPCK-treated trypsin or 50 μg/ml of proteinase K diluted in MEM/BSA. Subsequently, the cells were washed twice with ice cold MEM/BSA, one time 10 min with either 125 μg/ml soybean trypsin inhibitor or 2 mM phenylmethylsulfonyl fluoride dissolved in MEM/BSA. For morphological analysis the cells were subsequently rinsed with PBS containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ (PBS^+), and immediately fixed.

Before internalization of avidin-TRITC, avidin-gold, or avidin-HRP it was necessary to biotinylate the apical surface of the cells. All manipulations were performed at 4°C. Cells were washed three times with HBSS containing Ca^{2+} and Mg^{2+} salts and 20 mM Hepes, pH 7.4, ($HBSS^+$) and treated twice for 15 min with 0.5 mg/ml of NHS-LC-biotin (Pierce, Rockford, IL) dissolved in the same buffer. The cells were then washed twice quickly and one time for 10 min with MEM/BSA. In the immunofluorescence analysis NHS-SS-biotin was used, and following apical internalization of avidin-TRITC, the apical surface of the cells was stripped of biotin-avidin complexes by incubating the cells twice for 20 min in reducing solution. This reagent was prepared by dissolving 155 mg of reduced glutathione in 9.0 ml of 83 mM NaCl. Immediately prior to use 1 ml of calf serum and 60 μl of 50% (wt/vol) NaOH was added. Cells were washed with PBS^+ and immediately fixed.

Fixation and Fluorescent Labeling of Cells

Samples were fixed with paraformaldehyde using a pH-shift protocol (Bomsel et al., 1989) or with glutaraldehyde. In the pH-shift protocol, cells were fixed 5 min at room temperature with 4% paraformaldehyde, 80 mM Pipes/KOH, pH 6.5, 5 mM EGTA, 2.0 mM $MgCl_2$, and then transferred to 4% paraformaldehyde dissolved in 100 mM NaBorate, pH 11.0, and incubated 10 min at room temperature. Cells were washed 2 × 3 min with PBS, pH 8.0, and nonreacted paraformaldehyde was quenched 10 min at room temperature with 75 mM NH_4Cl, 20 mM Glycine, pH 8.0 (both dissolved in PBS, pH 8.0). Following two 5-min washes with PBS, pH 8.0, nonspecific sites were blocked with PBS, 0.7% (wt/vol) fish skin gelatin (FSG), and 0.025% (wt/vol) saponin (Sigma). In experiments in which the nucleus was stained with propidium iodide 100 μg/ml of boiled RNAse A was added to the blocking solution. In some experiments cells were fixed with 0.2% (vol/vol) glutaraldehyde dissolved in 80 mM Pipes/KOH, pH 6.8, 5 mM EGTA, 2.0 mM $MgCl_2$, for 10 min at room temperature. Nonreacted glutaraldehyde was quenched by incubating the cell three times 15 min with freshly prepared 1 mg/ml $NaBH_4$ dissolved in PBS, pH 8.0. The cells were washed twice with PBS and blocked in PBS-FSG-saponin as described above.

The fixed cells were incubated with the appropriate primary antibodies, diluted in PBS-FSG-saponin, for 45 min at 37°C in a humid chamber, and then washed 3 × 3 min with PBS-FSG-saponin, 1 × 3 min with PBS-saponin, and 1 × 3 min with PBS-FSG-saponin. Subsequently, the cells were incubated 45 min at 37°C with the appropriate combination of fluorescently labeled secondary antibodies and propidium iodide (2 μg/ml) diluted in PBS-FSG-saponin. The cells were washed 3 × 3 min with PBS-FSG-saponin, 1 × 3 min with PBS-saponin, and 2 × 3 min with PBS, pH 8.0, alone, 5 min with 0.1% Triton X-100, dissolved in PBS, pH 8.0, followed by a 5-min wash in PBS, pH 8.0, alone. Cells were postfixed in 4% paraformaldehyde dissolved in 100 mM Na-cacodylate, pH 7.4, for 30 min at room temperature, washed twice with PBS, and mounted in *p*-phenylene diamine (Sigma) (Johnson, 1981) which was prepared as follows: 2 ml of 200 mM Tris, pH 8.2, was added to 20 ml of glycerol and mixed by bubbling nitrogen gas through the solution for 20 min at room temperature. 200 milligrams of *p*-phenylene diamine was added and the mixing continued for two additional hours. The mounting medium was stable for 7–10 d when stored in a capped syringe at −20°C. The slides were stored at −20°C until viewing with the confocal microscope.

The specificity of the immunofluorescence staining was confirmed as follows:

IgA. No IgA signal was detected in cells in which this ligand was not internalized, or in MDCK cells not expressing the pIgR. The anti-rat, anti-mouse, and anti-rabbit secondary antibodies did not cross-react with the IgA, and the anti-human IgA secondary antibody did not cross-react with any of the other ligands, fluid-phase markers, antibodies, or secondary antibodies.

ZO-1, α-TfR, TGN-38, α-tubulin, and γ-tubulin. The specificity of staining for these molecules was based on their established distribution in these and other cells. None of these antibodies cross-reacted with IgA, and when omitted from the staining protocol no signal was detected. The distribution of these proteins was identical whether or not IgA was internalized.

Tf. No signal for Tf was detected in starved cells in which the ligand was not internalized or if Tf was added to the apical surface of the cells for 30 min at 37°C. In addition, in cells that had internalized Tf but were incubated with preimmune serum no labeling was detected. Labeling was not detected in cells in which the affinity purified anti-Tf antibodies were omitted and these antibodies did not cross-react with IgA. The distribution of Tf was identical whether or not IgA was internalized.

Scanning Laser Confocal Analysis of Fluorescently Labeled Cells

The samples were analyzed using a krypton-argon laser coupled with a Bio-Rad MRC600 confocal head, attached to an Optiphot II Nikon microscope with a Plan Apo 60X 1.4 NA objective lens. The samples were scanned simultaneously for FITC and Texas red (or propidium iodide or TRITC) emission using the K1 and K2 filter blocks. In each figure the left panel shows FITC fluorescence while the right shows Texas red, propidium iodide, or TRITC fluorescence. Collection parameters were as follows: zoom = 3.0 or 3.5, 0.5 s/scan, 5 frames/image, Kalman filter, motor step size = 0.5 μm, diaphragm closed or set at 1/3 open. The data was analyzed using Comos software and regions of colocalization were identified using the merge side function. The images were converted to tagged-information-file-format (TIFF) and the contrast levels of the images adjusted in the Photoshop program (Adobe Co., Mountain View, CA) on a Macintosh IIci (Apple, Cupertino, CA). The contrast-corrected images were imported into Pagemaker (Aldus Corporation, Seattle, WA) and printed from an Agfa 9,800 imagesetter at 2,400 dots per inch, using a line screen of 150 lines/inch.

Ultrastructural Analysis of IgA-HRP Transcytosis

Following internalization of IgA-HRP and avidin-gold, the cells were washed three times quickly with MEM/BSA and one time with ice cold PBS^+. The cells were immediately fixed by adding ice-cold 0.5% (vol/vol) glutaraldehyde in 200 mM Na cacodylate, pH 7.4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and incubating the cells for 30 min at room temperature. Cells were rinsed three times with 200 mM Na cacodylate buffer, pH 7.4, and 0.1% (wt/vol) diaminobenzidine (DAB), dissolved in 200 mM cacodylate buffer, was added for 2 min at room temperature. The DAB solution was aspirated and replaced with fresh DAB solution containing 0.01% (vol/vol) H_2O_2 and incubated 30 min at room temperature in the dark. Samples were rinsed with 200 mM Na cacodylate, pH 7.4, and osmicated with 1% OsO_4 (wt/vol), 200 mM Na cacodylate, pH 7.4, 1% (wt/vol) $K_4Fe(CN)_6$, for 90 min at 4°C. After several rinses with H_2O the samples were block-stained overnight with 0.5% (wt/vol) uranyl acetate in H_2O. Filters were dehydrated in a graded series of ethanol, embedded in the epoxy resin LX-112 (Ladd Res. Inds., Inc., Burlington, VT), and sectioned with a diamond knife (Diatome, Fort Washington, PA). Sections, 200–225-nm thick (as determined by their interference colors) were mounted on butvar-coated nickel grids and viewed at 80 kV in a Zeiss (West Germany) EM-10 electron microscope without further contrasting.

To confirm that IgA-HRP was efficiently transcytosed, this ligand was basolaterally internalized for 10 min at 37°C by pIgR-expressing cells. Following a 2-h chase at 37°C approximately 80% of IgA-HRP was found in the apical medium (transcytosed), 5% was released basolaterally, and the remainder was cell associated. IgA-HRP was not significantly internalized in the presence of 100-fold excess unlabeled IgA or by MDCK cells not expressing the pIgR. Similarly, no DAB reaction was detected at the EM level if an excess of IgA (2.5 mg/ml) was added during the internalization of the IgA-HRP to the cell surface, if the IgA-HRP was omitted during the inter-

nalization step, or if IgA–HRP was internalized by non-transfected MDCK cells.

Analysis of [^{125}I]Tf Recycling

Iron-saturated Tf was iodinated to a specific activity of 5.0–9.0 × 10^6 cpm/μg using ICl as described (Breitfeld et al., 1989*a*). The cells were depleted of endogenous Tf by incubating for 4 h in MEM/BSA and [^{125}I]Tf (5 μg/ml) was internalized from the basolateral surface of the cells for 2 h at 18°C. The cells were washed two times quickly and one time 3 min with MEM/BSA at 18°C before incubation at 37°C in the presence of 50 μg/ml of cold Tf. At the appointed times the cells were rapidly cooled on ice and the apical and basolateral media were collected. [^{125}I]Tf was stripped from the cell surface by incubating the cells for 60 min at 4°C with 750 mM Glycine, pH 2.5, diluted 1:5 with PBS$^+$. The Transwells were rinsed with PBS$^+$ and the filters were cut out of their holders. The total [^{125}I]Tf initially bound to the cells includes ligand recycled to the basolateral surface, ligand transcytosed into the apical medium, ligand stripped from the cell surface with acid, and cell associated ligand not sensitive to glycine stripping (endocytosed), and was quantitated in a gamma counter (Beckman Instruments, Palo Alto, CA). [^{125}I]Tf uptake was inhibited >95% when the radioactive ligand was internalized in the presence of a 100-fold excess of cold ligand.

Analysis of [^{125}I]IgA Endocytosis and Postendocytotic Fate

[^{125}I]IgA was iodinated using the ICl method to a specific activity of 1.0–2.0 × 10^7 cpm/μg. Endocytosis of [^{125}I]IgA was measured as described (Breitfeld et al., 1989*b*). When specified 25 μg/ml of trypsin was included in the apical medium. At the conclusion of the experiment [^{125}I]IgA was stripped from the cell surface and the results quantitated as described above for the analysis of [^{125}I]Tf recycling. The postendocytotic fate of a preinternalized cohort of [^{125}I]IgA was analyzed as described (Breitfeld et al., 1989*b*). When specified 25 μg/ml of trypsin was included in the apical medium.

DAB Density-shift Assay

In the original DAB density-shift assay, cells were homogenized following internalization of markers (one of which was conjugated to HRP and the other radiolabeled) and a fraction rich in endosomal markers was purified by centrifugation (Courtoy et al., 1984). During homogenization there was always some breakage of endosomes. This resulted in loss of radiolabeled signal and the release of HRP conjugate that could non-specifically cross-link vesicles when these fractions were reacted with DAB and H_2O_2. Following the advice of J. Kaplan (University of Utah, Salt Lake City, UT), we have modified this original protocol, by omitting the homogenization step and performing the DAB reaction on whole cells. After internalization of [^{125}I]Tf (5 μg/ml), [^{125}I]IgA (5 μg/ml), Fab–HRP (25 μg/ml), or avidin–HRP (25 μg/ml) the cells were washed with ice-cold MEM/BSA and radiolabeled ligands stripped from the cell surface with 100 μg/ml trypsin (in the case of [^{125}I]IgA) or with 50 μg/ml proteinase K (in the case of [^{125}I]Tf) for 3 × 10 min at 4°C. The cells were then washed twice with ice-cold HBSS$^+$. DAB reaction buffer (0.5 ml) was added to both apical and basal compartments of the Transwell. This reagent was prepared by adding 3.3 ml of 3 mg/ml of DAB (dissolved in HBSS$^+$, pH adjusted to 7.4 with NaOH, and filtered), and 20 μl of 30% (vol/vol) H_2O_2 to 20 ml of HBSS$^+$. In control reactions H_2O_2 was omitted from the DAB reaction buffer. Following a 45-min incubation at 4°C the cells were washed twice with HBSS$^+$, the filters were carefully excised from their holders, boiled for 2 min in 0.4 ml of SDS lysis buffer (0.5% [w/v] SDS, 100 mM triethanolamine, pH 8.6, 5 mM EDTA, 0.02% [wt/vol] NaN_3), and vortex shaken for 15 min at 4°C. Under these conditions <5% of the total counts were associated with the filter. The supernatants were then centrifuged at 100,000 *g* in a Sorvall RP70AT rotor for 25 min at 20°C. Radioactivity was quantitated in a gamma counter.

In this assay radiolabeled ligand present in the avidin–HRP or Fab–HRP filled apical endosomal compartment is cross-linked by the DAB reaction into a dense, detergent-insoluble complex that is recovered in the pellet following centrifugation. The absolute value for the amount of ligand present in the DAB cross-linked endosomes was obtained by dividing the amount of ligand present in the pellet by the total amount of ligand present in both the pellet and supernatant. The percent of ligand that pelleted when H_2O_2 was omitted from the DAB reaction was subtracted from this value (typically these values were less than 5% of the total counts). Finally these values were normalized to those obtained when [^{125}I]IgA and Avidin–HRP or Fab–HRP were cointernalized for 10 min at 37°C or 120 min at 18°C.

Results

Accumulation of IgA in a Subapical Compartment of pIgR-expressing MDCK Cells

In Fig. 2 we have analyzed the time and temperature requirements for basolaterally internalized IgA to accumulate at the apical pole of pIgR-expressing MDCK cells. Individual sections, obtained with a confocal microscope, are shown from the basal portion of the cell below the nucleus (level *4*), from the lateral surface of the cell at the level of the nucleus (level *3*), from the apical region of the cell above the nucleus (level *2*), and from the apical pole of the cell at or above the level of the tight junctions (level *1*).

Following a 5-min pulse at 37°C basolaterally internalized IgA was found in discrete vesicles below, at the level of, and above the nucleus (Fig. 2 *A*). At the level of the tight junctions the majority of the IgA remained at the cell margins, however, some IgA was also found in apical elements at the apex of the cell. When the 5-min pulse was followed by a 10-min chase, IgA was found throughout the cell including a large accumulation of ligand apically in both clusters and as small individual vesicles that were typically centrally distributed and radiated towards the margins of the cell (Fig. 2 *B*). Following a 25-min chase little IgA could be found in the basal and lateral portions of the cell (Fig. 2 *C*). Instead, the IgA was largely distributed in vesicular elements present in the apical cytoplasm above the nucleus and at the apex of the cell. Following the 25-min chase the intensity of IgA staining had decreased and by 90 min of chase little IgA could be detected in the cell (Fig. 2 *D*). These results demonstrate that IgA moves rapidly from peripheral basolateral endosomes and subsequently accumulates at the apical pole of the cell as it is being transcytosed. The distribution of the pIgR was almost identical to its ligand; it was found throughout the cell but was especially concentrated at the apical pole of the cell.

The distribution of IgA in cells incubated at 18°C was also assessed. At this temperature IgA can be recycled basolaterally, however, release of the ligand from the apical pole of the cell is inhibited (Hunziker et al., 1990). Following a 30-min incubation at 18°C the IgA was found in a peripheral endosomal compartment that lies close to the basolateral cell surface (Fig. 2 *e*). Although similar to the distribution seen after 5 min at 37°C, at 18°C there was little IgA located above the nucleus. After 2 h of internalization, IgA was also found not only in the peripheral endosomes but also throughout the cytoplasm (Fig. 2 *f*). At the level of the tight junctions some IgA was also found concentrated in a centralized spot of bright fluorescence. This cluster of IgA was often more compact and condensed than observed at 37°C and may reflect alterations in the interaction of this compartment with the cytoskeleton at 18°C. This distribution is similar to that reported by Hunziker et al. (Hunziker et al., 1990). The distribution of IgA at 2 h was not altered if the incubation period was extended to 4 h (not shown). Like at 37°C, IgA internalized for 30 min at 18°C was translocated to the apical pole of the cell when the cells were chased in ligand free medium for 90 min at 18°C (not shown).

Transcytosing IgA Accumulates in Apical Endosomes

We were next interested in determining the nature of the apical compartment in which the IgA was accumulating. We considered three possibilities: the apically distributed IgA could represent transcytotic vesicles that had not yet fused with the apical plasma membrane, or IgA that had reached the apical surface and had been re-internalized into apical endosomes, or IgA that was delivered directly from basolateral to apical endosomes. To distinguish among these three possibilities we determined if the basolaterally internalized IgA that accumulates in the apical compartment is accessible to apically internalized ligands. A prediction of the classical model for transcytosis presented in Fig. 1 is that transcytotic vesicles would be inaccessible to apically internalized ligands. If, however, the apically distributed IgA was accessible to apically internalized ligands it might suggest that one of the latter possibilities was true.

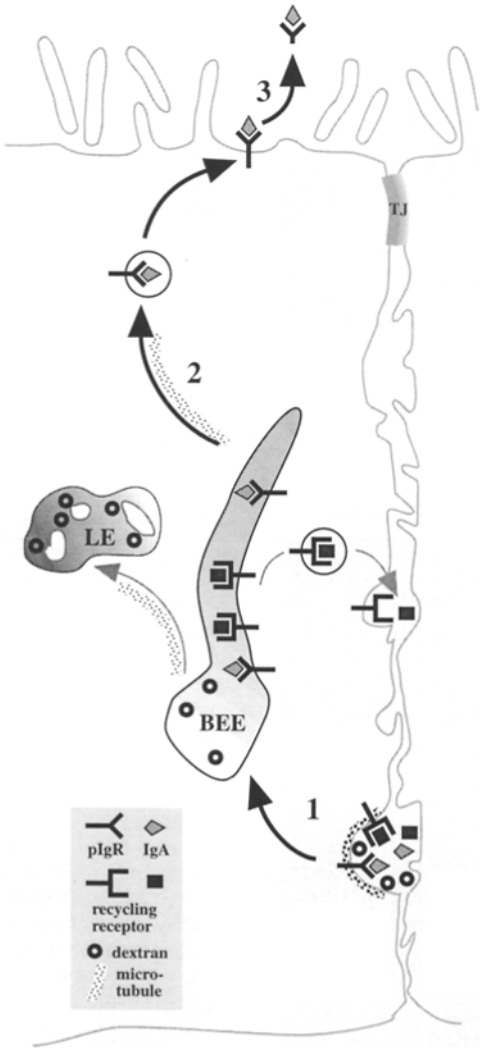


Figure 1. Classical model for transcytosis of the pIgR in MDCK cells. In the classical model for transcytosis IgA is internalized, along with recycling and fluid-phase molecules, into a common basolateral early endosome (step *1*). Within this endosomal compartment several sorting events occur. Fluid-phase markers are delivered to late endosomes, while many receptors (e.g., Tf-receptor) are recycled back the basolateral plasma membrane. IgA is packaged into transcytotic vesicles (step *2*), which ultimately fuse with the apical plasma membrane. At the apical cell surface a proteinase cleaves the receptor to SC, releasing it and bound IgA into secretions (step *3*). *BEE*, basolateral early endosome; *LE*, late endosome/prelysosome; *TJ*, tight junction.

In the first part of our analysis we have labeled apical endosomes with monovalent anti-SC Fab fragments (derived from affinity purified anti-SC antibodies). When added apically the anti-SC Fab fragments act as a pseudoligand for the pIgR and are efficiently internalized and recycled from the apical cell surface (>95% of this ligand is recycled apically) (Breitfeld et al., 1989*b*). Little of this ligand is transcytosed in the apical-basolateral direction. As demonstrated in Fig. 3 *a* there was a significant degree of colocalization of basolaterally internalized IgA with Fab fragments internalized apically for 10 min at 37°C. Colocalization of the two ligands was also apparent in endosomal structures that lay below the level of the tight junctions and above the nucleus (not shown). Of course at the light level it is not possible to rule out that IgA and Fab fragments are segregated into separate subdomains of an apically distributed compartment. However, using a density-shift assay (described below) we estimate that approximately 75% of the IgA is present in this Fab-labeled apical endosomal compartment (see Fig. 6 *b* for quantitation). In this and many of the subsequent experiments IgA was continuously internalized for 30 min at 37°C to completely label all of the IgA-accessible compartments brightly. The distribution of IgA internalized for 30 min at 37°C is like that shown in Fig. 2 *b*, with the majority of IgA present in the apical region of the cell and some present in peripheral basolateral structures. Similar results were obtained if cells were pulse labeled with IgA for 5 min and subsequently chased prior to the addition of apical ligand.

Identical results were observed if Fab fragments were internalized from the basolateral surface and IgA was internalized apically (not shown). Similarly, the meeting of apically added Fab fragments with basolaterally internalized IgA occurred in cells incubated at 18°C (Fig. 3 *b*), a temperature at which transcytosis is inhibited. The significant degree of colocalization observed at 37° and 18°C suggests that the apically accumulated IgA did not represent unfused transcytotic vesicles, but rather an endosomal compartment that was accessible to both basolaterally internalized IgA and apically internalized ligands.

IgA Is Delivered to Apical Endosomes Under Conditions Where Apical Endocytosis of the Ligand Is Prevented

The apical accumulation of IgA we observed could be the result of ligand that was first delivered to the apical plasma membrane and then internalized into an apical endosomal compartment. It is known that cleavage of the pIgR to SC is slow relative to apical endocytosis; consequently, the pIgR and its bound ligand can be endocytosed from the apical cell surface (Breitfeld et al., 1989*b*). If this is the case it should be possible to prevent IgA from being internalized from the apical cell surface by including trypsin in the apical medium. We have previously used trypsin to cleave pIgR molecules as they arrive at the basolateral surface of the cell, thereby, preventing their subsequent endocytosis and transcytosis

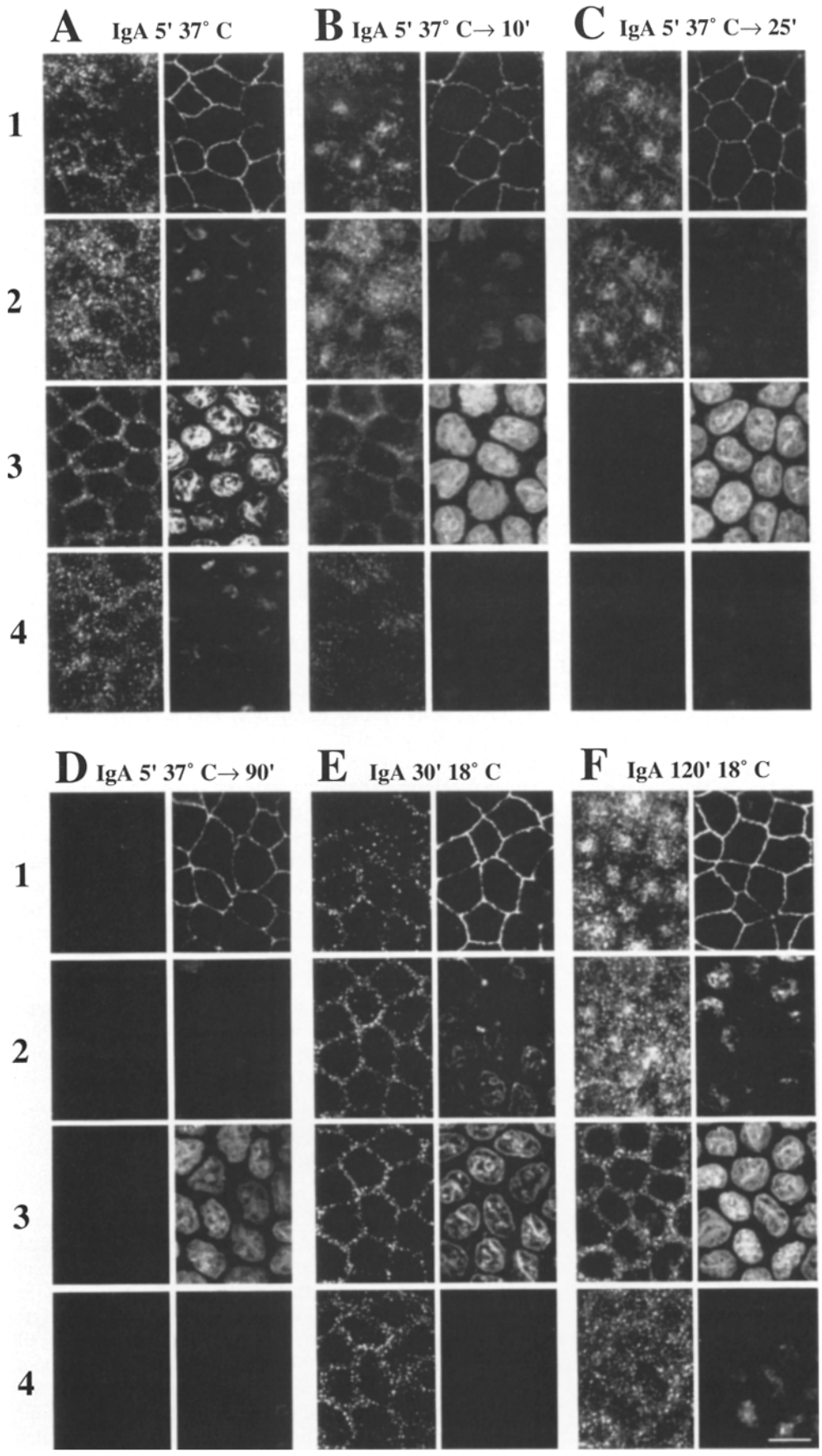


Figure 2. Time and temperature dependence of IgA accumulation in a subapical compartment of pIgR-expressing MDCK cells. IgA was internalized from the basolateral surface of MDCK cells expressing the pIgR for 5 min at 37°C (*a*). Following the 5-min pulse of IgA the cells were rapidly washed and chased in the absence of ligand for 10 (*b*), 25 (*c*), or 90 min at 37°C (*d*). IgA was also internalized basolaterally for 30 (*e*) or 120 min at 18°C (*f*). At the end of the internalization period the cells were washed and ligand bound to cell surface receptors stripped with trypsin at 4°C. The cells were fixed with paraformaldehyde and stained for IgA, for the tight junction protein ZO-1, and for the nucleus. Individual sections, obtained with a scanning laser confocal microscope, are shown from the basal portion of the cell below the nucleus (level *4*), from the lateral surface of the cell at the level of the nucleus (level *3*), from the apical region of the cell above the nucleus (level *2*), and from the apical pole of the cell at or above the level of the tight junctions (level *1*). The samples were scanned simultaneously for FITC and Texas red (or propidium iodide) emission which are displayed in the left and right halves of each panel, respectively. All images are at the same magnification. Bar, 10 μm.

(Casanova et al., 1991; Aroeti et al., 1993). In contrast, if IgA is delivered directly from basolateral to apical endosomes then addition of apical trypsin should have little effect on the ability of the IgA to enter the apical endosomal compartment.

To distinguish between the two possibilities described above we first needed to confirm that trypsin treatment could be used to prevent internalization of IgA from the apical cell surface. [^{125}I]IgA was prebound to pIgR molecules at the apical cell surface at 4°C, and then the cells were warmed up for 1–5 min at 37°C in the absence or presence of 25 μg/ml of trypsin. As shown in Fig. 4 *a*, in the absence of trypsin, the [^{125}I]IgA was internalized, albeit inefficiently, during the 37°C incubation. However, in the presence of trypsin no internalized [^{125}I]IgA was detected during the same 5-min internalization period (Fig. 4 *b*). In addition, the vast majority of IgA (>95%) was rapidly released into the apical medium even after a 1-min incubation period in the presence of the proteinase. These observations demonstrate that trypsin is able to rapidly remove IgA bound to pIgR from the cell surface before it has a chance to be internalized, and that the binding of IgA to the pIgR does not render the pIgR insensitive to trypsin treatment.

In addition, we have analyzed the effect of apical trypsin on the fate of a 5-min pulse of basolaterally internalized [^{125}I]IgA. As is shown in Fig. 4 *c* the addition of trypsin increases the initial rate of ligand transcytosis over that observed in the absence of proteinase. This observation suggests that in the absence of trypsin a fraction of the transcytosing IgA is being reinternalized from the apical cell surface and presumably recycling while in transit to the apical secretions of the cell. It also points out the importance of using apical trypsin to prevent reinternalization of the pIgR from the apical cell surface.

Having confirmed that trypsin could be effectively used to prevent ligand internalization we determined the effect of apical trypsin on IgA accumulation in the apical compartment. IgA was internalized continuously from the basolateral surface of the cell while at the same time trypsin was included in the apical medium. As shown in Fig. 4 (*d* and *e*) the delivery of IgA to the apical compartment of these trypsin-treated cells occurred largely undiminished at either 37° or 18°C, respectively. These results suggested that under these conditions much of the IgA was being delivered directly to this apical compartment. We have also performed the analysis described in Fig. 2 (*a–d*) in the presence of apical trypsin. Similar results were obtained, although as expected there was a more rapid decrease in IgA signal (data not shown). As an additional control for the effectiveness of the apical trypsin treatment in preventing internalization of pIgR present at the apical plasma membrane, Fab fragments were added in conjunction with the trypsin to the apical surface of the cells shown in Fig. 4 (*d* and *e*). Under these conditions internalization of Fab fragments was completely prevented. Compare untreated cells in the left panel of Fig. 3 *a* with trypsin-treated cells in the left panel of Fig. 4 *d*.

We have performed several additional controls to verify the effectiveness of the apical trypsin in preventing IgA–pIgR complexes from being internalized from the apical plasma membrane. First, identical results were obtained using as little as 5 μg/ml trypsin, indicating that we were working in considerable excess of the trypsin needed to cleave the pIgR. Second, Fab fragments bind to pIgR–IgA complexes (Lemaître-Coelho et al., 1981; and Bomsel, M., and K. E. Mostov, unpublished results), so if pIgR–IgA complexes transiently appeared at the apical surface before reinternalization, the Fab fragments could still bind. Third, when analyzed by PAGE, trypsin-treated [^{125}I]Fab fragments remained intact and were not degraded (not shown). In addition, if soybean trypsin inhibitor was added to the trypsin–Fab medium after the incubation at 37° or 18°C, the Fab fragments were rapidly internalized to the same extent as non-trypsin–treated Fabs (not shown). This indicates that the effect of trypsin is to inactivate the ability of the pIgR to bind Fab fragments, and not to inactivate the Fab fragments.

To confirm that IgA was being delivered to an apical en-

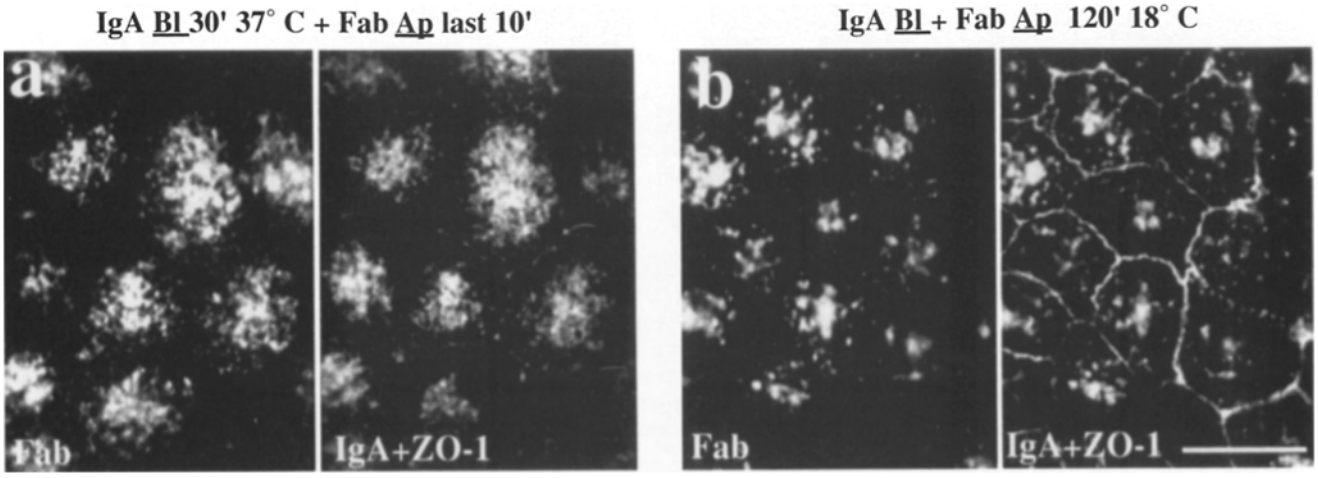


Figure 3. IgA is delivered to an apical endosomal compartment. IgA was internalized from the basolateral surface of the cells for 30 min at 37°C and anti-SC Fab fragments were simultaneously internalized apically during the last 10 min of the incubation period (*a*). Alternatively, IgA was internalized basolaterally and Fab fragments apically for 120 min at 18°C (*b*). At the end of the experiment the cells were washed and cell surface ligand stripped from the apical cell surface with trypsin. Cells were fixed with paraformaldehyde, stained with the appropriate antibodies, and scanned simultaneously for FITC and Texas red emission which are displayed in the left and right halves of each panel, respectively. Sections are at or above the level of the tight junctions. Images are at the same magnification. Bar, 10 μm.

dosomal compartment under conditions where apical internalization of the ligand is prevented, we have performed the following experiments. IgA was internalized basolaterally for 20 min at 37°C. The apical surface of the cell was biotinylated and avidin–TRITC was added apically while IgA was internalized basolaterally for an additional 10 min. Trypsin was included in the apical medium throughout the 30-min internalization period. The internalized avidin–TRITC serves as a general membrane marker for the apical endosomal compartment and unlike Fab fragments internalization of avidin–TRITC is not prevented by the action of trypsin, as the pool of biotinylated proteins (and lipids) is largely trypsin resistant. The 10-min internalization period is long enough to fill the apical early endosomal compartment but short enough to prevent entry of the avidin–TRITC into the prelysosomal compartment (Bomsel et al., 1989; Parton et al., 1989). Under these conditions there was significant colocalization of the two ligands in the apical region of the cell, both at the level of the tight junctions (Fig. 5 *a*), and in the apical cytoplasm below the level of the tight junctions (Fig. 5 *b*). Avidin–TRITC was not detected below this level (data not shown). In addition, colocalization of the two markers was observed if the period of internalization of the two markers was limited to just 10 min at 37°C (data not shown), confirming that a large fraction of the IgA was rapidly being delivered directly to an apical early endosomal compartment.

To demonstrate that colocalization of IgA and avidin was occurring at the ultrastructural level, IgA–HRP was internalized for a total period of 30 min at 37°C, while avidin–gold was internalized for the final 10 min from the biotinylated apical pole of the cell. Apical trypsin was included through-

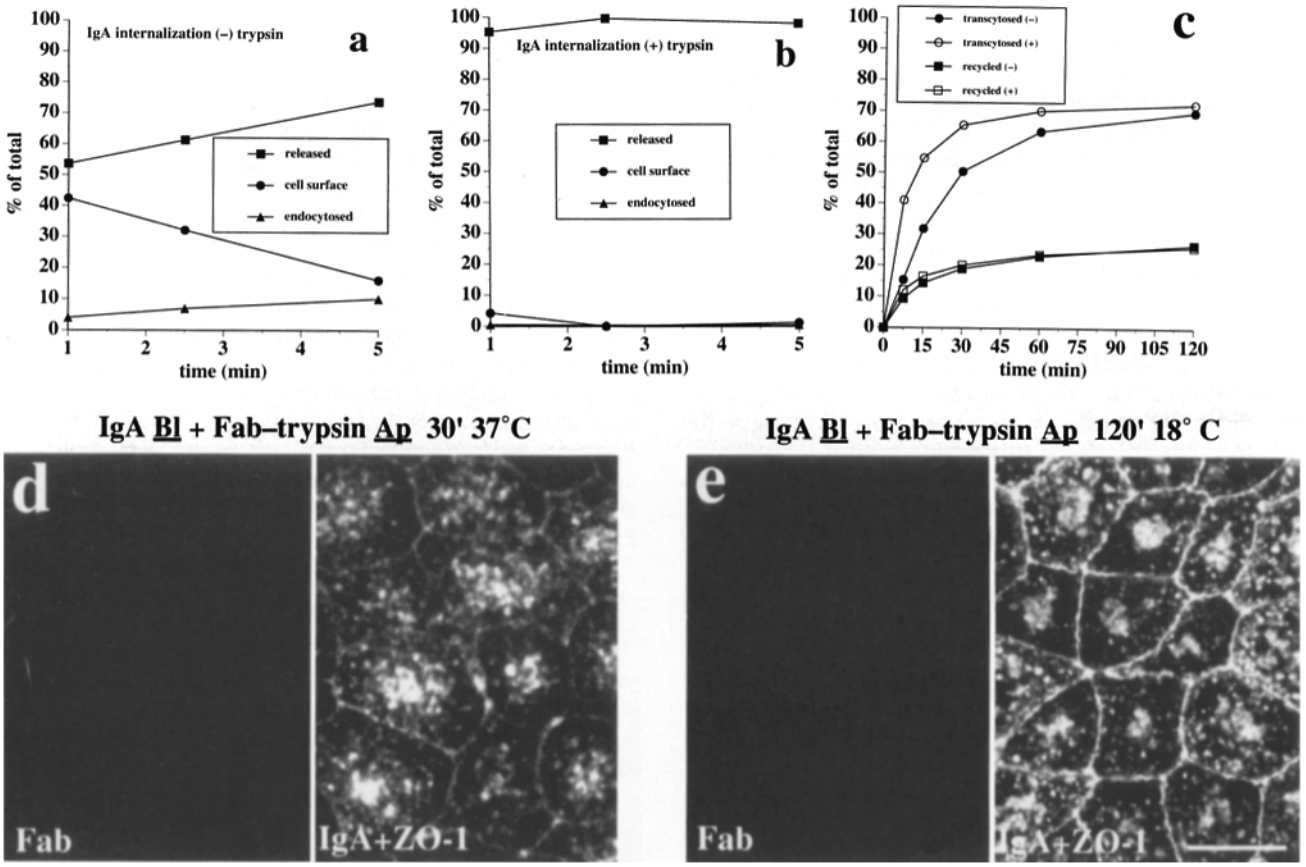


Figure 4. Trypsin prevents internalization of IgA from the apical cell surface. In *a* and *b* [^{125}I]IgA was bound to the apical surface of cells for 90 min at 4°C, the cells were washed, and then reincubated at 37°C in the absence (*a*) or presence (*b*) of 25 μg/ml of trypsin added to the apical chamber of the Transwell. At the indicated times the cells were rapidly cooled on ice and the apical media was collected. [^{125}I]IgA was stripped from the cell surface by using acid treatment at 4°C and the filters were cut out of their holders. The total [^{125}I]IgA initially bound to the cells included ligand released into the apical medium, ligand stripped from the cell surface with acid, and cell-associated ligand not sensitive to stripping (*endocytosed*), and was quantitated in a gamma counter. Values are from duplicate filters and varied <10%. At time 0, prior to warming the cells up to 37°C, virtually 100% of the ligand was at the cell surface. In *c* [^{125}I]IgA was internalized from the basolateral surface of the cells for 5 min at 37°C, the cells were washed, and then chased for 120 min in the absence (–) or presence of (+) apical trypsin. The percent of total ligand released apically (*transcytosed*) and basolaterally (*recycled*) are shown. The remainder of the counts were intracellular. Values are from duplicate filters and varied <10%. In *d–e* IgA was internalized basolaterally for 30 min at 37°C or 120 min at 18°C, respectively, while Fab fragments plus 25 μg/ml of trypsin were added apically. Cells were fixed with paraformaldehyde, stained with the appropriate antibodies, and scanned simultaneously for FITC and Texas red emission which are displayed in the left and right halves of each panel, respectively. *d* and *e* are projections (sums) of four sections from the apical pole of the cell. Use of projections gives one an overview of the distribution of a particular marker in a single image. Images are at the same magnification. Bar, 10 μm.

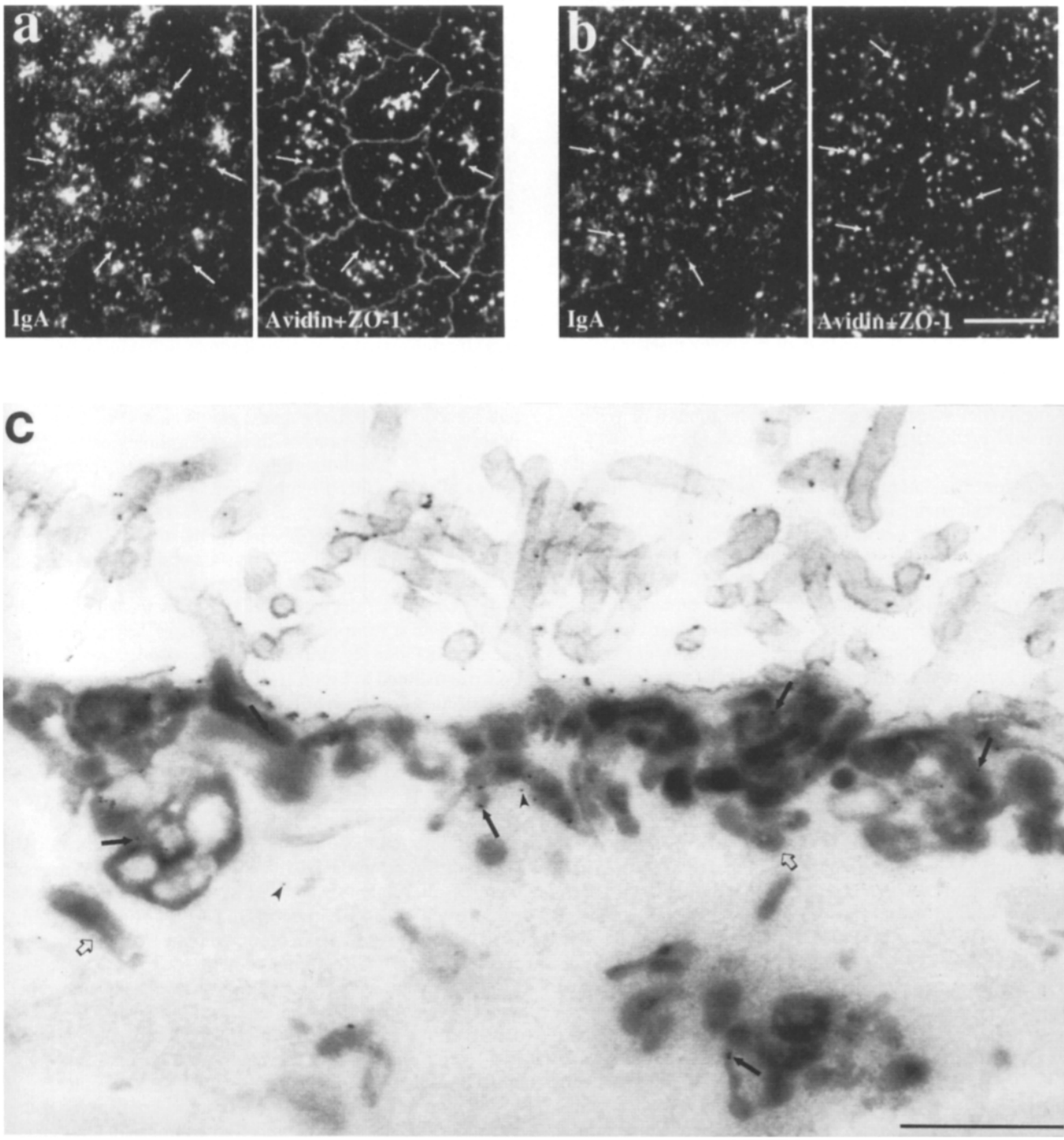


Figure 5. IgA is delivered to an apical endosomal compartment under conditions that prevent internalization of the ligand from the apical cell surface. In *a* and *b* IgA was internalized basolaterally for 30 min at 37°C while avidin-TRITC was added during the last 10 min of the incubation period to the biotinylated apical pole of the cell. Trypsin was included in the apical pole of the cell throughout the internalization period. At the end of the experiment cell surface avidin-SS-biotin complexes were stripped from the cell surface with reduced glutathione. Cells were fixed with paraformaldehyde, stained with the appropriate antibodies, and scanned simultaneously for FITC and TRITC emission which are displayed in the left and right halves of each panel, respectively. *a* is a section at the level of the tight junctions. *b* is a section above the nucleus, approximately 2-μm below the level of the tight junctions. Arrows are intended as landmarks to guide the reader in identifying regions of colocalization. All images are at the same magnification. In *c* IgA–HRP was internalized from the basolateral cell surface for 30 min at 37°C. Avidin–gold, bound to the biotinylated apical pole of the cell, was internalized apically during the last 10 min. Trypsin was included in the apical medium throughout the internalization period. Meeting was observed in tubulovesicular elements close to the apical cell surface. Cells were processed for electron microscopy as described in the Materials and Methods. Solid arrows, examples of structures in which IgA–HRP and avidin–gold colocalize; open arrows, examples of structures in which only IgA–HRP is found; arrowheads, examples of areas where avidin–gold is found in the absence of IgA–HRP. Bars: (*a* and *b*) 10 μm; (*c*) 0.5 μm.

out the 30-min internalization period. The IgA–HRP was detected by DAB cytochemistry. IgA–HRP was found in vesicular and tubular elements distributed throughout the cytoplasm but concentrated in the apical region of the cells when semi-thick sections (200–225 nm) of epon-embedded cells were examined. Particularly in the apical region of the cell, just under the apical plasma membrane, there were large accumulations of short tubules that contained both avidin–gold and IgA–HRP (Fig. 5 *c*, *solid arrows*). This observation confirms the IgA–HRP is entering an apical early endosomal compartment. Colocalization also occurred in more vesicular organelles close to the plasma membrane (Fig. 5 *c*) and occasionally in perinuclear multivesicular bodies.

Although it was possible to find regions of IgA–HRP that had no gold (Fig. 5 *c*, *open arrows*), it was less common to find avidin–gold in the absence of the other marker (Fig. 5 *c*, *arrowheads*). We believe this reflects the inefficient internalization of the avidin-gold particles. In addition, some of the IgA–HRP structures could represent tubules or vesicles derived from basolateral early endosomes not yet fused with the apical endosomal compartment, recycling vesicles derived from basolateral endosomes, or some other unidentified compartment. In these examples >90% of the avidin–gold particles are found to colocalize with the IgA–HRP. Avidin–gold was not detected in the basal portions of the cell. Similar results were obtained if the two markers were internalized for 120 min at 18°C (data not shown). In addition, we observe significant colocalization between IgA–HRP and apically internalized ricin–gold (data not shown) which is a non-specific membrane marker that is efficiently internalized and recycled at the apical cell surface (van Deurs et al., 1990).

Biochemical Estimation of the Amount of IgA Delivered to the Apical Endosomal Compartment

To quantitate the amount of IgA delivered directly to apical endosomes we have used a modification of the DAB density-shift protocol (Courtoy et al., 1984). In our assay [^{125}I]IgA was internalized basolaterally for a total period of 30 min at 37°C to completely fill all of the IgA-accessible compartments. Avidin-HRP was internalized during the last 10 min of the incubation period from the biotinylated apical pole of the cell. Apical trypsin was included throughout the internalization period. At the end of the experiment, ligand was removed from the cell surface, and the cells were treated with DAB and H_2O_2. When cells are treated in this manner, [^{125}I]IgA present in the avidin–HRP filled apical endosomal compartment is cross-linked by the DAB reaction into a dense, detergent-insoluble complex. When the cells are solubilized in SDS the [^{125}I]IgA present in DAB cross-linked apical endosomes is recovered by centrifugation. [^{125}I]IgA present in other compartments remains soluble and is found in the supernatant following centrifugation. The percent of IgA in the pellet represents that fraction of IgA present in the apical endosomal compartment. It is well established that DAB cross-linking is not 100% efficient, even for markers that are completely colocalized (Courtoy, 1984; Ajioka, 1986; Ward et al., 1990). As such our values were normalized to reactions in which IgA and avidin–HRP were co-internalized from the biotinylated apical pole of the cell (see legend to Fig. 6 for these values). Under these conditions we expect maximum colocalization of the two markers, and indeed observe a high degree of colocalization of the two markers in apical endosomes (Fig. 6 *a*). Our maximal levels for colocalization, as determined by the HRP cross-linking assay, are comparable to or even better than those obtained in many similar studies (Ajioka, 1986; Ward et al., 1990).

When we normalize to the maximum amount of [^{125}I]IgA found in the apical endosomal compartment, we estimate that 80.5 ± 2.39% of [^{125}I]IgA internalized for 30 min at 37°C resides in an apical endosomal compartment (Fig. 6 *b*). This result is consistent with our morphological analysis in which the majority of transcytosing IgA resides in the apical region of the cell in a compartment that overlaps with apical endosomes. Moreover, we obtain a very similar result when [^{125}I]IgA is internalized basolaterally and avidin–HRP apically for only 10 min at 37°C (>80%). In addition, [^{125}I]IgA was also delivered to apical endosomes following a 2-h internalization period at 18°C. Under these particular conditions we estimate that approximately 34.4 ± 2.0% of the ligand had been delivered to the apical endosomes (Fig. 6 *b*). This observation is consistent with our morphological evidence (see Fig. 2 *f*) that although IgA is delivered to apical endosomes at this reduced temperature, a large fraction of the ligand remains in basolateral compartments that are presumably inaccessible to apically internalized ligand. The lower level of colocalization at 18°C may also reflect a less efficient internalization of ligand that occurs at 18°C.

As a control for the validity of our assay we have also performed both a morphological analysis of IgA transcytosis and the HRP density-shift assay in nocodazole-treated cells. As demonstrated in Fig. 6 (*c–d*) meeting of basolaterally internalized IgA and apically internalized Fab fragments was prevented in nocodazole-treated cells. As expected, the IgA remained along the basolateral cell surface (Fig. 6 *c*) and was not delivered to the cytoplasm at the apical pole of the cell (Fig. 6 *d*). The Fab fragments were found dispersed across the apical region of the cell in small vesicles (Fig. 6 *d*, *left*). These results confirm previous observations that the translocation of IgA from the basolateral to apical pole of the cell requires microtubules, and is largely inhibited in nocodazole-treated cells (Breitfeld et al., 1990; Hunziker et al., 1990). Similar results were observed if the experiments were performed at 37°C (data not shown).

As predicted, entry of [^{125}I]IgA into apical endosomes was inhibited sevenfold at both 37° and 18°C when the DAB-density shift assay was performed in nocodazole-treated cells (Fig. 6 *b*). Again, these estimates were normalized to the maximum amount of [^{125}I]IgA found in the apical endosomal compartment. This observation confirms that the majority of IgA was being delivered from basolateral endosomes to apical endosomes, and that the process was microtubule dependent. In addition, the sevenfold inhibition of meeting in drug-treated cells confirms that [^{125}I]IgA was only density shifted when both the [^{125}I]IgA and avidin–HRP were in the same compartment. Note that endocytosis from both poles of the cell is largely unaffected by drug treatment and the amount of [^{125}I]IgA internalized by the nocadozole-treated cells actually increased (data not shown) (Breitfeld et al., 1990; Hunziker et al., 1990).

As additional controls for the specificity of the DAB density-shift assay we report that there was no shift when avidin–HRP was not internalized, and when [^{125}I]IgA and avidin–HRP were bound to opposite poles of the cell at 4°C,

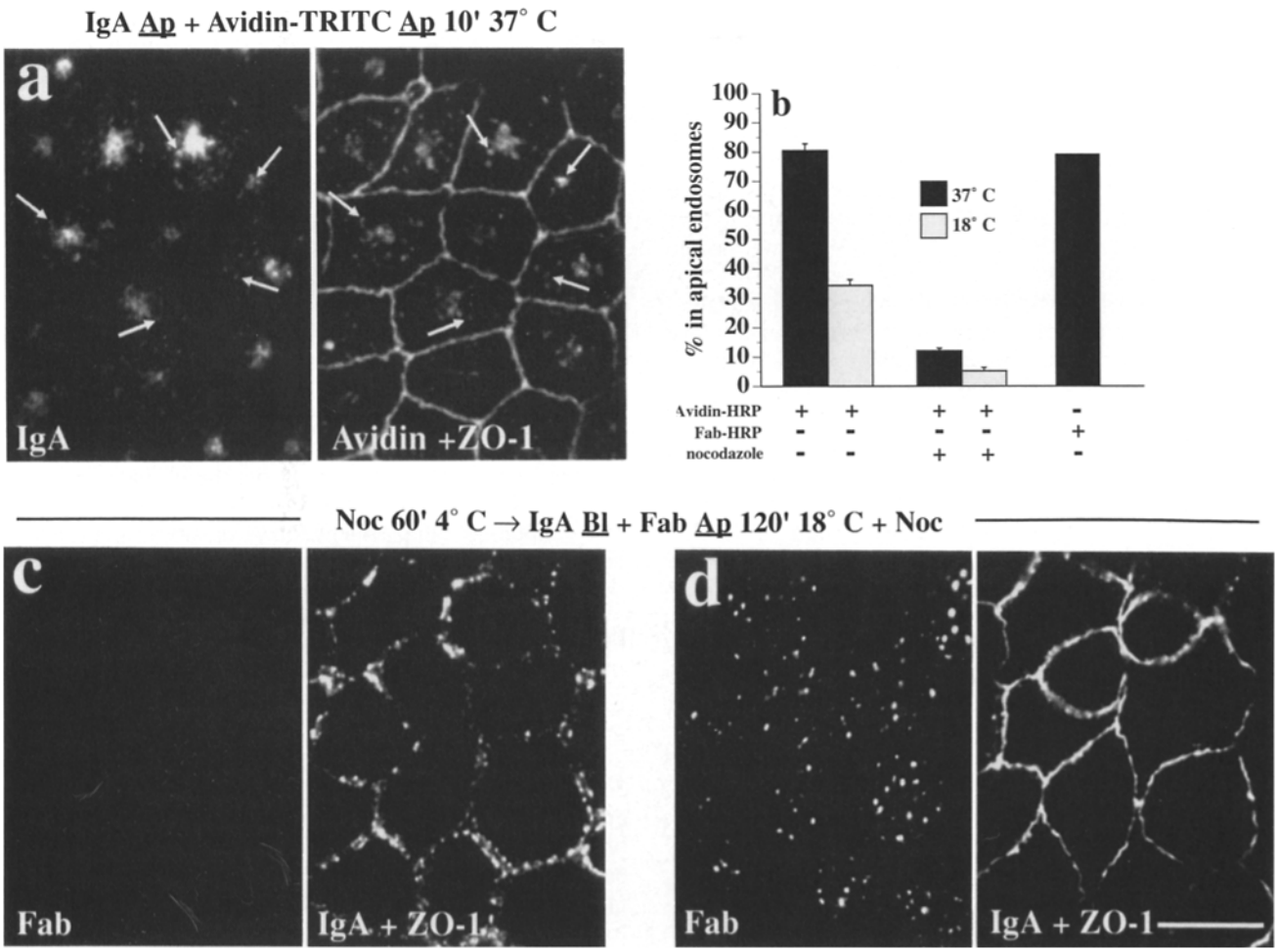


Figure 6. Quantitation of the amount of [^{125}I]IgA delivered to apical endosomes. In *a* IgA and avidin-TRITC were co-internalized from the apical surface of the cell for 10 min at 37°C. At the end of the experiment cell surface avidin-SS–biotin complexes were stripped from the cell surface with reduced glutathione, and the cells were processed for immunofluorescence. A projection of the three most apical sections are shown. Arrows are intended as landmarks to guide the reader in identifying regions of colocalization. In *b* ligands were internalized as follows: [^{125}I]IgA was internalized for 20 min at 37°C, the cells were cooled down and the apical cell surface biotinylated at 4°C. Avidin–HRP was prebound to the apical cell surface for 15 min at 4°C. The cells were then allowed to internalize [^{125}I]IgA basolaterally and avidin–HRP apically for an additional 10 min at 37°C. Apical trypsin was included throughout the internalization period. To measure meeting at 18°C the cells were pretreated for 15 min at 37°C with apical trypsin, the apical cell surface was biotinylated at 4°C, and [^{125}I]IgA was internalized basolaterally and avidin–HRP apically for 120 min at 18°C. In one series of experiments, [^{125}I]IgA was internalized for 30 min at 37°C, and during the last 10 min of ligand internalization Fab–HRP was added apically for 10 min at 37°C. When specified, cells were preincubated with nocodazole for 60 min at 4°C and the drug was included in all subsequent steps. Details of the DAB reaction and quantitation are given in the Materials and Methods. Results are mean ± SD ($n \geq 3$). Values for cointernalization, used for normalization of the data, were as follows: [^{125}I]IgA and avidin–HRP 10 min at 37°C, 41.2 ± 1.3%; [^{125}I]IgA and avidin–HRP 120 min at 18°C, 44.8 ± 6.0%; [^{125}I]IgA and Fab–HRP 10 min at 37°C, 57.3 ± 8.0%; [^{125}I]IgA and Fab–HRP 120 min at 18°C, 50.8 ± 0.1%. In *c–d* cells were treated with nocodazole 60 min at 4°C and then IgA was internalized basolaterally and Fab fragments apically for 120 min at 18°C in the continued presence of the drug. Following trypsin treatment cells were processed for immunofluorescence. Sections are shown from the lateral surfaces of the cell (*c*) and at the level of the tight junctions (*d*). Bar, 10 μm.

only 1.2 ± 0.9% of the [^{125}I]IgA was pelletable. Like the nocodazole control, the latter observation confirms that [^{125}I]IgA is not density shifted unless it is in the same compartment as the avidin–HRP, and that the [^{125}I]IgA does not nonspecifically associate with the DAB-polymerized apical endosomes subsequent to the lysis of the cells in SDS.

The Release of IgA into Secretions Occurs from Apical Endosomes

The above data is consistent with our hypothesis that IgA is delivered from basolateral to apical endosomes. Two further lines of evidence confirm that the subsequent release of IgA into secretions occurs from the apical endosomal structures we have described. First, as shown in Fig. 2 *c* when a 5-min pulse of IgA is followed by a 25-min chase at 37°C the large majority of IgA is distributed at the apical pole of the cell. Careful examination of Fig. 4 *c* indicates that in the interval between 25 and 120 min, IgA that was in the compartment defined by Fig. 2 *c* is released predominantly from the apical pole of the cell, i.e., three times more IgA is released api-

cally than basolaterally. This confirms that IgA present in the apical compartment of the cell is largely on the transcytotic pathway.

Finally, we have performed the following experiment. [^{125}I]IgA was internalized from the basolateral surface of the cell for 30 min at 37°C in the presence of apical trypsin. As described above under these conditions the majority of [^{125}I]IgA resides in an apical endosomal compartment. The cells were then nocodazole treated at 4°C. When warmed up to 37°C in the continued presence of the drug only [^{125}I]IgA present in compartments distal to the peripheral basolateral endosomes, e.g., the apical endosomal compartment, will be capable of being released into the apical medium (Hunziker et al., 1990). As demonstrated in Fig. 7 *a* the majority of [^{125}I]IgA was released apically under these conditions. To confirm that release of IgA was occurring from the apical endosomes we have analyzed at the morphological level the distribution of IgA in cells treated identically. As shown in Fig. 7 (*b–d*) following a 60-min chase at 37°C little of the IgA was present in the apical region of the cell. As expected some of the IgA remained in the basolateral endosomes as its delivery to apical endosomes was blocked by nocodazole. These observations are consistent with our findings in the DAB density-shift assay that the majority of IgA resides in an apical endosomal compartment and that IgA is released predominantly from this compartment.

Apically and Basolaterally Internalized Fluid-phase Markers Are Not Concentrated in the Apical Endosomal Compartment to Which IgA Is Delivered

The meeting of basolaterally endocytosed IgA with apically endocytosed avidin is reminiscent of fluid phase ligands, which when internalized from opposite poles of the cell converge in a perinuclear prelysosomal compartment (Bomsel et al., 1989; Parton et al., 1989). However, it appears that the compartment we have characterized is distinct from prelysosomes because as described below neither basolaterally nor apically internalized fluid-phase markers colocalize with IgA in the tubular subapical endosomal compartment. In the first set of experiments, IgA was internalized basolaterally for 30 min at 37°C and FITC–dextran was added continuously from the apical surface. The FITC–dextran was not found along the lateral surface of the cell at the level of the nucleus (Fig. 8 *a*), but was highly concentrated in the apical cytoplasm above the nucleus (Fig. 8 *b*). In the apical IgA compartment, which in this group of cells occurs above the level of the tight junctions, the fluid phase marker was generally excluded from the membrane proximal tubular aspects of the IgA-containing compartment (Fig. 8 *c*). Identical results were found after a 2-h incubation at 18°C (not shown). In addition, similar results were obtained when IgA (or Fab fragments) were cointernalized with FITC–dextran from the apical pole of the cell. Under these conditions there was little colocalization of the two markers in the subapical IgA containing compartment, however, above the nucleus colocalization of the two markers was observed (data not shown).

In addition we have determined if IgA colocalizes with basolaterally internalized FITC–dextran. When endocytosed basolaterally this fluid phase marker has access to peripheral basolateral early endosomes as well as perinuclear prelysosomes and lysosomes (Bomsel et al., 1989; Parton et al., 1989). FITC–dextran was internalized basolaterally for 3 h at 37°C to label both early and late elements of the endosomal pathway followed by an additional 30-min incubation in which IgA and FITC–dextran were cointernalized basolaterally. The IgA and FITC–dextran were colocalized in the basal portions of the cell (Fig. 8 *d*) and to some extent in the cytoplasm above the nucleus (Fig. 8 *e*). However, at the level of the tight junctions, where the IgA was found in abundance, little FITC–dextran was observed (Fig. 8 *f*). Identical results were observed at 18°C (not shown).

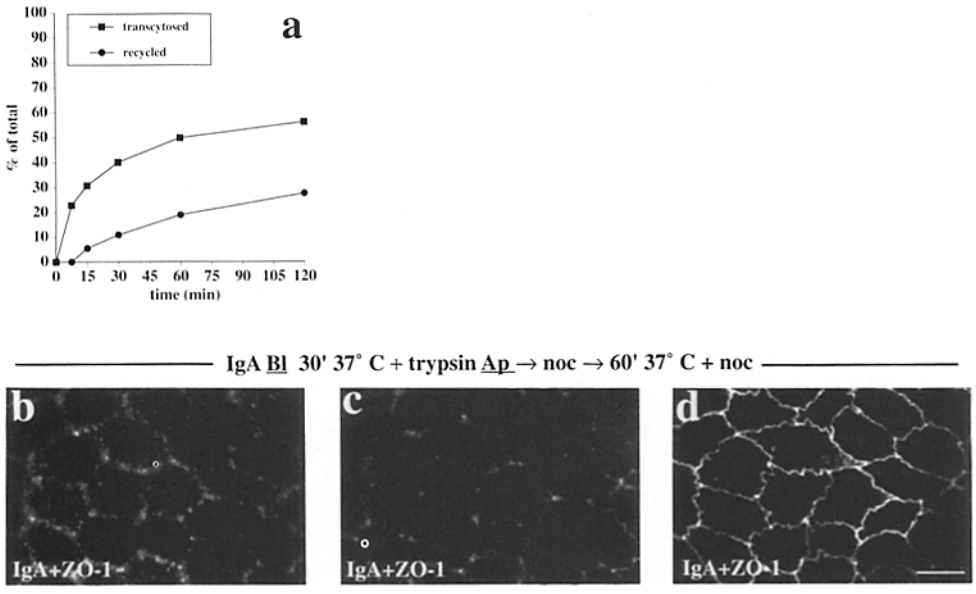


Figure 7. IgA is released into secretions from the apical endosomes of nocodazole-treated cells. In *a* [^{125}I]IgA was internalized from the basolateral surface of the cells for 30 min at 37°C in the presence of apical trypsin. The cells were treated at 4°C with nocodazole and then incubated 120 min at 37°C in the continued presence of the drug. The percent of total ligand released apically (transcytosed) and basolaterally (recycled) are shown. The remainder of the counts were intracellular. Values are from duplicate filters and varied <10%. In *b–d* nonradioactive IgA was internalized as described in *a*. Following nocodazole treatment the cells were chased 60 min at 37°C with the drug. Cells were fixed and stained for both IgA and ZO-1 using Texas red–labeled secondary antibodies. Sections are shown from the lateral cell surfaces (*b*), above the level of the nucleus (*c*), and at the level of the tight junctions (*d*). All images are at the same magnification. Bar, 10 μm.

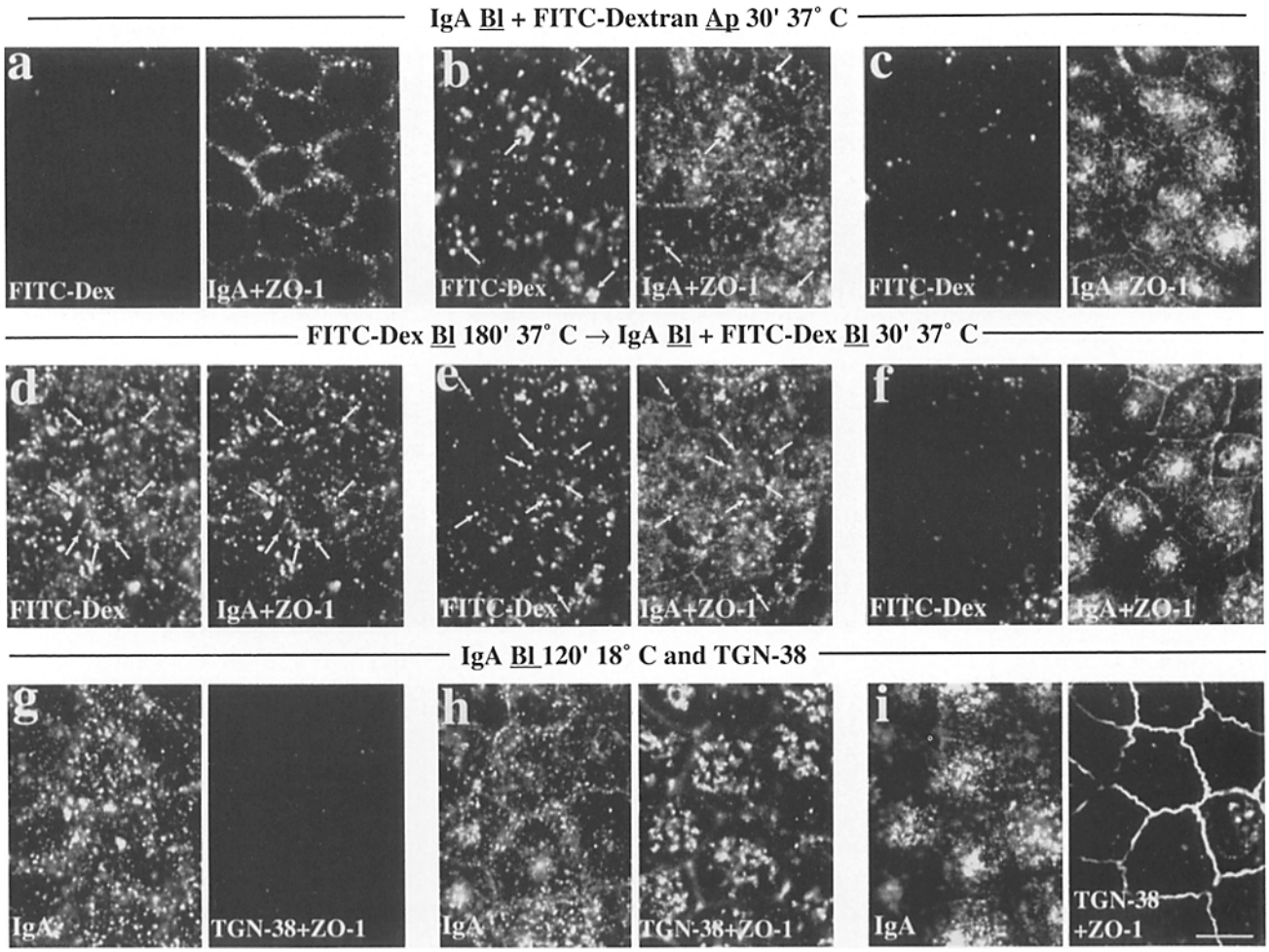
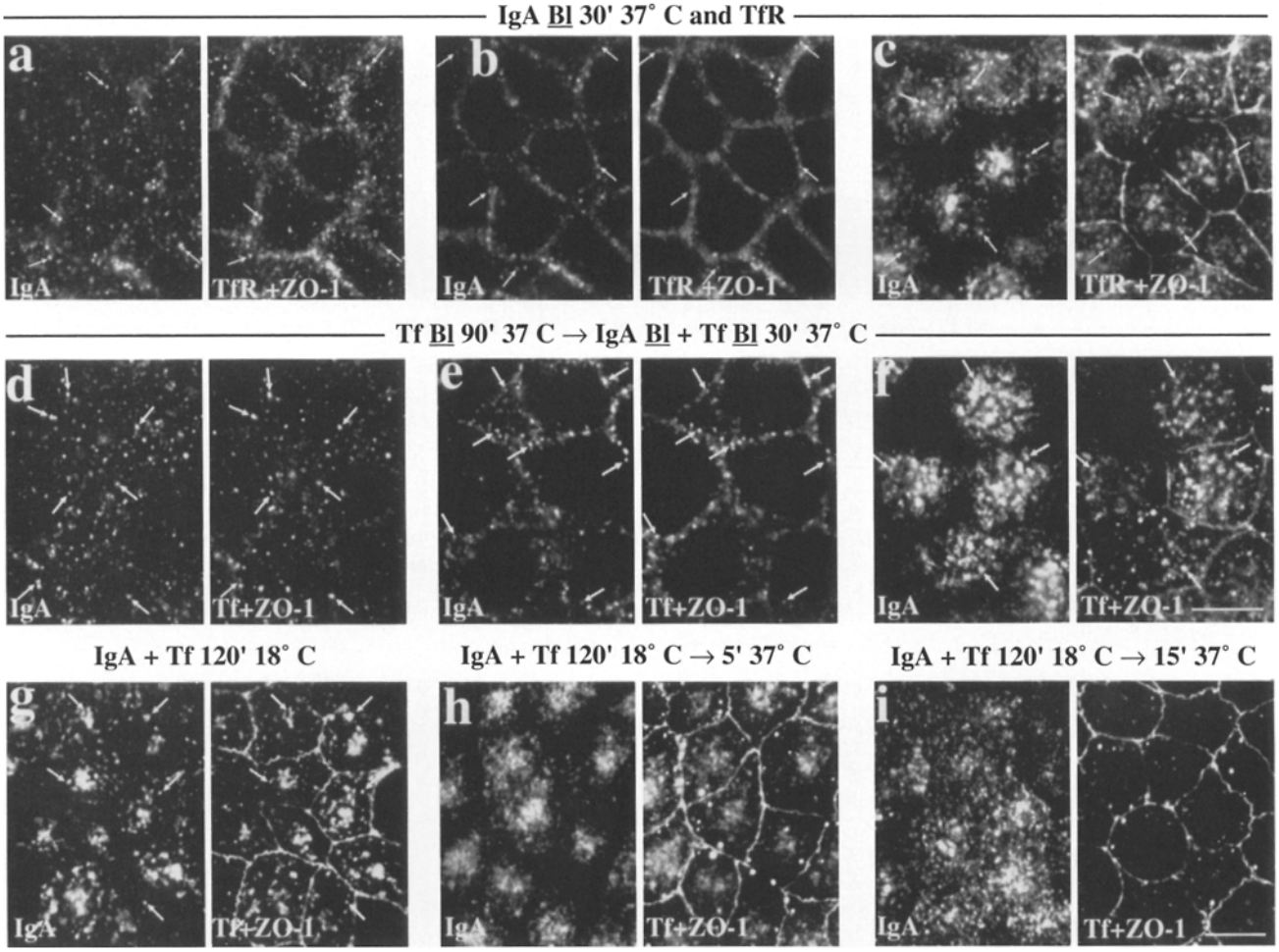


Figure 8. Apically and basolaterally internalized FITC–dextran and TGN-38 do not colocalize with IgA in the apical endosomal compartment. In *a–c* IgA was internalized basolaterally for 30 min at 37°C while FITC–dextran was internalized apically. Sections from the lateral surface of the cell, above the nucleus, and at the level of the tight junctions are shown in *a–c*, respectively. In *d–f* FITC–dextran was internalized basolaterally for 3 h at 37°C and IgA and FITC–dextran were subsequently internalized for an additional 30 min at 37°C. Sections from the basal portion of the cell (*d*) , the area above the nucleus (*e*), and at the level of the tight junctions (*f*) are shown. Staining for IgA, internalized for 120 min at 18°C, and TGN-38 is shown in *g–i*. Sections from the basal portion of the cell (*g*), the region above the nucleus (*h*), and at the level of the tight junctions (*i*) are shown. Samples were scanned simultaneously for FITC and Texas red emission which are displayed in the left and right halves of each panel, respectively. Arrows are intended as landmarks to guide the reader in identifying regions of colocalization. All images are at the same magnification. Bar, 10 μm.

The above results suggest that the apical IgA meeting compartment is distinct from the prelysosomal compartment where fluid-phase ligands meet. In addition we have localized transcytosing IgA and the AC17 antigen, a 95,000-M_r lysosomal membrane glycoprotein, that is thought to be delivered from basolateral early endosomes to late endosomes (Nabi et al., 1991). Like the fluid phase marker, the AC17 antigen was found predominantly in the basolateral and supranuclear portions of the cell, and although there was some staining at the level of the tight junctions, there was little colocalization of this antigen and apical IgA (not shown). Finally, some proteins are delivered from endosomes to the TGN. However the subapical compartment containing IgA does not appear to colocalize with TGN-38, a TGN resident protein (Luzio et al., 1990; Wilde et al., 1992). This protein was found predominantly above the nucleus (Fig. 8 *h*) but below the level of the tight junctions (Fig. 8 *i*).

Tf Has Access to an Apical Endosomal Compartment and Is Recycled Back to the Basolateral Cell Surface

Hughson and Hopkins have reported that extensive mixing of basolaterally internalized Tf–HRP and an apically internalized lectin, concanavalin-A, occurs in an apical endocytic compartment of the intestinal epithelial cell line Caco-2 (Hughson and Hopkins, 1990). We were interested in determining if Tf and the Tf-receptor, both of which recycle at the basolateral surface of MDCK cells, would enter the apical endosomal compartment in these cells. We have first compared the distribution of IgA with the total compartment that the Tf-receptor has access to at steady-state. It is apparent in Fig. 9 (*a–c*), that there is large amount of overlap between basolaterally internalized IgA and the Tf-receptor, especially in the apical most region of the cell (Fig. 9 *c*). Like IgA, there was a significant degree of colocalization of Tf-



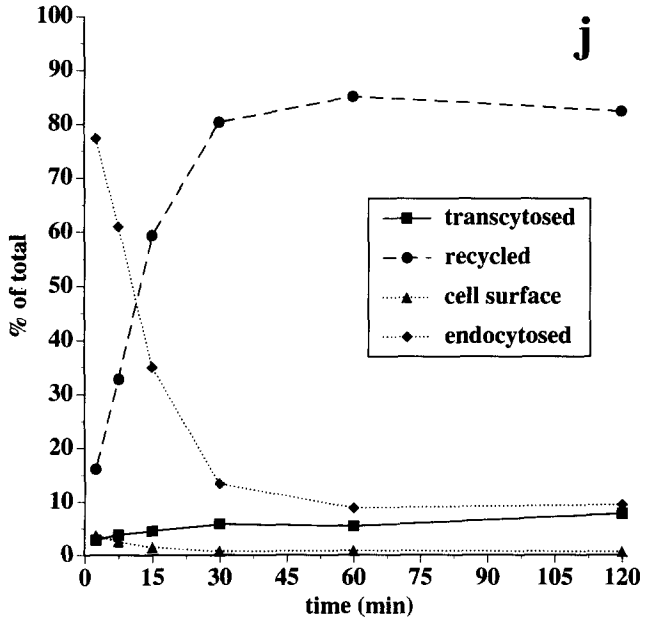


Figure 9. Basolaterally endocytosed transferrin reaches the apical compartment where IgA accumulates and recycles to the basolateral cell surface. In *a–c* cells were stained for IgA internalized for 30 min at 37°C and Tf-receptor. Sections are shown from the basal region of the cell (*a*), from the lateral portion of the cell (*b*), and a sum of three sections from the apical pole of the cell (*c*). In *d–f* Tf was internalized basolaterally for 90 min at 37°C and IgA and Tf were subsequently internalized basolaterally for an additional 30 min at 37°C. Sections are shown from the basal region of the cell (*d*), from the lateral portion of the cell (*e*), and at the level of the tight junctions (*f*). In *g–i* IgA and Tf were internalized basolaterally for 120 min at 18°C, washed, and then incubated in ligand free medium for 0 (*g*), 5 (*h*), or 15 min (*i*), at 37°C. Sections are from the apical region of the cell, at the level of the tight junctions. Samples were trypsin treated at 4°C, fixed with paraformaldehyde, stained, and scanned simultaneously for FITC and Texas red emission which are displayed in the left and right halves of each panel, respectively. Arrows are intended as landmarks to guide the reader in identifying regions of colocalization. *d–f* are shown at a higher magnification than *a–c* and *g–i*. In *j* [^{125}I]Tf was internalized from the basolateral surface of cells for 2 h at 18°C, the cells were washed, and then reincubated in the absence of radioactive ligand at 37°C. At the indicated times the cells were rapidly cooled on ice and the apical and basolateral media were collected. [^{125}I]Tf was stripped from the cell surface by using acid treatment at 4°C and the filters were cut out of their holders. The total [^{125}I]Tf initially bound to the cells included ligand recycled to the basolateral medium, ligand transcytosed into the apical medium, ligand stripped from the cell surface with acid, and cell associated ligand not sensitive to stripping (endocytosed), and was quantitated in a gamma counter. Values are from duplicate filters and varied <10%. Bars, 10 μm.

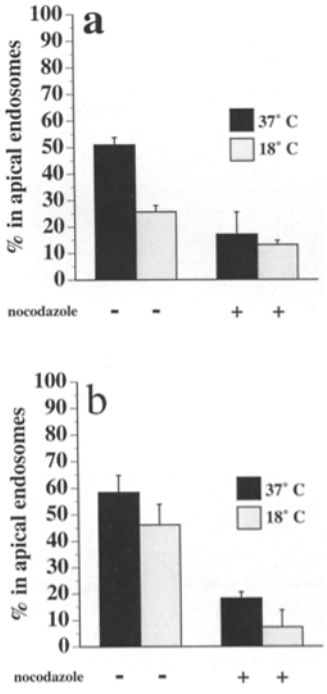


Figure 10. Quantitation of the amount of [^{125}I]Tf delivered to apical endosomes. In *a* [^{125}I]Tf was internalized basolaterally for 90 min at 37°C, and trypsin was added to the apical surface of the cell during the last 15 min of the internalization. The cells were cooled down and the apical cell surface biotinylated at 4°C. Avidin–HRP was prebound to the apical cell surface for 15 min at 4°C. The cells were then allowed to internalize [^{125}I]Tf basolaterally and avidin–HRP apically for an additional 10 min at 37°C. To measure meeting at 18°C the cells were pretreated for 15 min at 37°C with apical trypsin, the apical cell surface was biotinylated at 4°C, and [^{125}I]Tf was internalized basolaterally and avidin–HRP apically for 120 min at 18°C. In *b* [^{125}I]Tf was internalized for 90 min at 37°C. During the last 10 min of ligand internalization Fab–HRP was added apically for 10 min at 37°C. At 18°C [^{125}I]Tf was added basolaterally and Fab–HRP was internalized apically for 120 min at 18°C. When specified, cells were preincubated with nocodazole for 60 min at 4°C and the drug was included in all subsequent steps. Details of the DAB reaction and quantitation are given in Materials and Methods. Results are mean ± SD ($N \geq 3$). Values for cointernalization, used for normalization of the data, are given in the legend of Fig. 6.

receptor and apically endocytosed Fab fragments under these conditions (not shown).

In addition, we have analyzed the distribution of Tf to determine if it also colocalizes with IgA in the apical endosomal compartment. Tf was internalized basolaterally for 90 min at 37°C to fill the Tf-containing compartment. Subsequently, IgA and Tf were added for an additional 30 min at 37°C. There was colocalization of the two ligands in the basal (Fig. 9 *d*) and lateral (Fig. 9 *e*) regions of the cell. Although both ligands were found at the apex of the cell, colocalization of the two ligands was apparent in only limited regions of this area of the cell (Fig. 9 *f*). Our results suggest that there may be a good deal of overlap between the IgA and Tf-receptor compartments, however, at 37°C there is apparently segregation of the Tf and IgA ligands in the apical region of the cell. A possible explanation for why the overlap of IgA with the Tf-receptor was more clearcut is that binding of Tf to its receptor causes the receptor–ligand complex to be more rapidly segregated from the IgA.

Colocalization of Tf and IgA was more dramatic at the apical pole of the cell when both ligands were simultaneously internalized for 2 h at 18°C (Fig. 9 *g*). This probably reflects the reduced rate of recycling that occurs at 20°C (Hughson and Hopkins, 1990), and suggests that under these conditions less rapid segregation of the two ligands occurs. The Tf that accumulates in the apical region of the cell following the internalization at 18°C was removed from this compartment when the cells were warmed up to 37°C in the absence of added Tf. After 5 min at 37°C there was substantial colocalization of the IgA and Tf (Fig. 9 *h*), but by 15 min at 37°C little Tf was detected in the apical or other regions of the cell (Fig. 9 *i*). These results demonstrate that Tf can be sorted away from IgA in the apical endosomal compartment.

The following experiment confirms that Tf was efficiently sorted from the IgA present in apical endosomes. [^{125}I]Tf was internalized for 120 min at 18°C to allow the ligand access to all compartments of the cell including apical endosomes. Efficiency of basolateral recycling of the ligand was assessed upon reculture of the cells at 37°C. As shown in Fig. 9 *j*, [^{125}I]Tf was rapidly recycled back to the basolateral surface of the cell. These kinetics are similar to those reported for [^{125}I]Tf preinternalized for 60 min at 37°C (Podbilewicz and Mellman, 1990). Even though Tf was distributed in the apical region of the cell at 18°C, greater than 85% of the [^{125}I]Tf was rapidly recycled basolaterally, and less than 5% of the ligand was released apically. The above results confirm that the apical endosomal compartment we have characterized is capable of efficiently recycling Tf back to the basolateral cell surface.

In addition, we have used the DAB density-shift protocol to estimate the amount of Tf present in the apical endosomal compartment. The values for Tf were normalized to the maximal values obtained when IgA and avidin were cointernalized from the apical surface of the cell to facilitate comparison of the Tf and IgA data. As shown in Fig. 10 *a* we estimate that 51.0 ± 2.8% of the [^{125}I]Tf resides in an apical endosomal compartment following a 90-min pulse of the ligand at 37°C. At 18°C we estimate that 25.5 ± 2.4% of this ligand was found in avidin–HRP-labeled apical endosomes. When analyzed morphologically, the translocation of Tf from the basolateral endosomes to the apical region of the cell is prevented in nocodazole-treated cells (data not shown). As expected, nocodazole treatment inhibited the ability of [^{125}I]Tf to enter the apical endosomal compartment, reducing entry of [^{125}I]Tf into this compartment threefold at 37°C. When [^{125}I]Tf and avidin–HRP were bound to opposite poles of the cell at 4°C only 1.9 ± 0.6% of this ligand was density shifted.

As additional evidence for the presence of Tf in apical endosomes, we have also determined the amount of [^{125}I]Tf present in apical endosomes labeled with the pIgR-ligand Fab–HRP. As shown in Fig. 10 *b* we estimate that 58.4 ± 6.4% of the [^{125}I]Tf resides in this compartment at 37°C, and 46.1 ± 7.8% at 18°C. As above, nocodazole treatment inhibits [^{125}I]Tf from entering the apical endosomal compartment. Results using either Fab–HRP or Avidin–HRP suggest that a large fraction of Tf resides in an apical endosomal compartment. The larger value obtained when Fab–HRP was used may reflect the more efficient internalization and recycling of this particular ligand. Furthermore the results with the Fab–HRP suggest that Tf has access to the same compartment that the pIg-R is recycling through at the apical cell surface.

The Organization of the Apical Endosomal Compartment Is Dependent on Microtubules

As described in Fig. 6 *d* above, when Fab fragments were internalized from the apical pole of nocodazole-treated cells

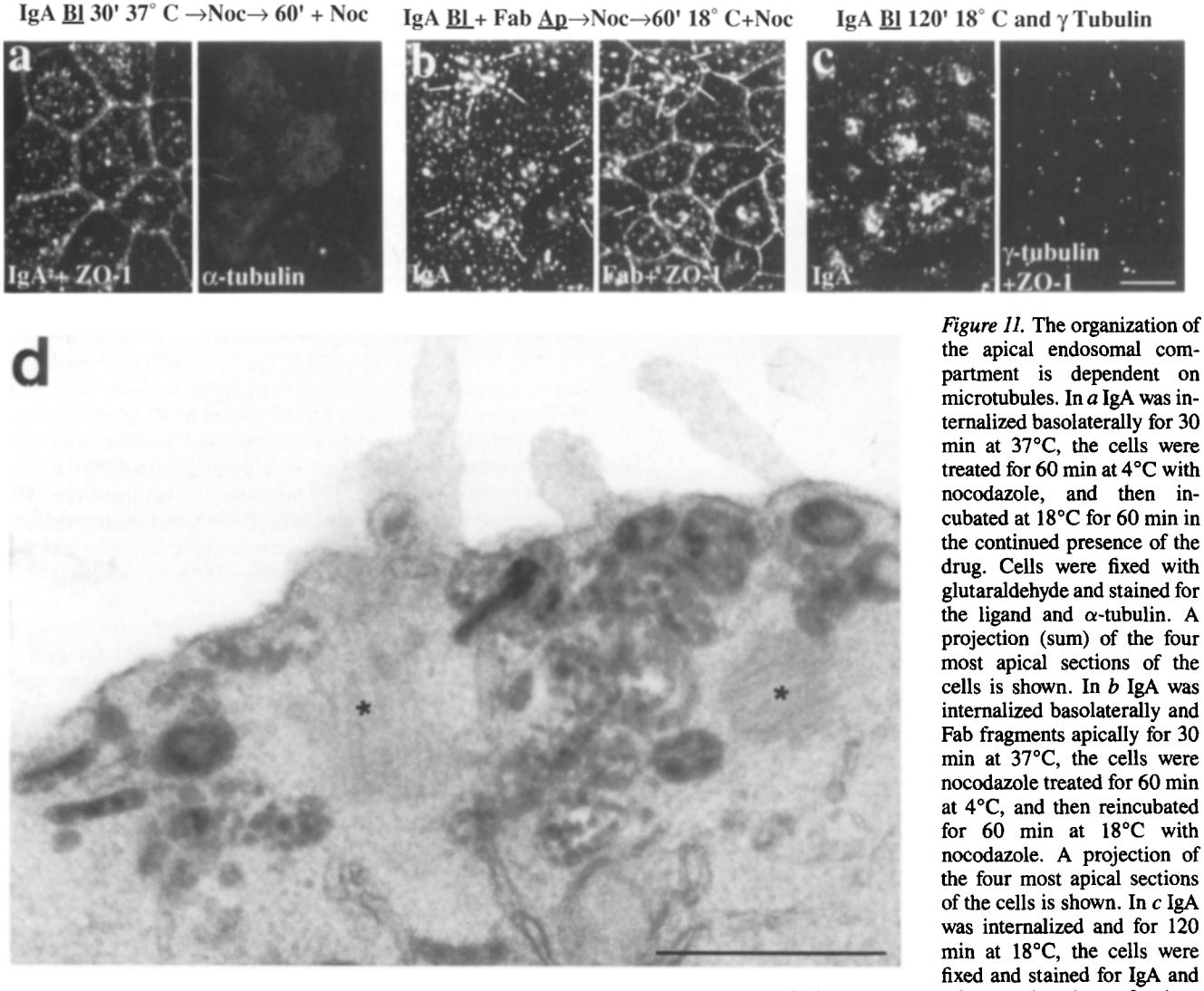


Figure 11. The organization of the apical endosomal compartment is dependent on microtubules. In *a* IgA was internalized basolaterally for 30 min at 37°C, the cells were treated for 60 min at 4°C with nocodazole, and then incubated at 18°C for 60 min in the continued presence of the drug. Cells were fixed with glutaraldehyde and stained for the ligand and α-tubulin. A projection (sum) of the four most apical sections of the cells is shown. In *b* IgA was internalized basolaterally and Fab fragments apically for 30 min at 37°C, the cells were nocodazole treated for 60 min at 4°C, and then reincubated for 60 min at 18°C with nocodazole. A projection of the four most apical sections of the cells is shown. In *c* IgA was internalized and for 120 min at 18°C, the cells were fixed and stained for IgA and γ-tubulin. This panel is a projection of the four most apical sections of the cell. The cells in *b* were treated with trypsin prior to fixation. Samples were scanned simultaneously for FITC and Texas red emission which are displayed in the left and right halves of each panel, respectively. Arrows are intended as landmarks to guide the reader in identifying regions of colocalization. All images are at the same magnification. In *d* IgA–HRP was internalized from the basolateral cell surface for 30 min at 37°C. Cells were processed for electron microscopy as described in the Materials and Methods. The centrioles are marked with an "*". Bars: (*a–c*) 10 μm; (*d*) 0.5 μm.

they were not found concentrated in the center of the cell but instead were dispersed in small vesicles across the apical pole of the cell. This observation suggested that the organization of the apical endosomal compartment was dependent on microtubules. To confirm this observation, IgA was internalized from the basolateral cell surface for 30 min at 37°C to load the tubulovesicular apical compartment with this ligand. Microtubules were depolymerized by treating the cells for 60 min at 4°C with nocodazole, and the cells were reincubated at 18°C in the continued presence of the drug. (Incubation was performed at 18°C to reduce the amount of IgA released apically.) Under these conditions the microtubular network was completely depolymerized (Fig. 11 *a*, *right*) and IgA was found in small vesicles dispersed across the apical cell cytoplasm (Fig. 11 *a*, *left*). The dispersion of IgA in the nocodazole-treated cells was not a result of cooling the cells to 4°C and rewarming to 18°C. In cells treated identically, but in the absence of the drug, the IgA was found in its centralized apical distribution typical of incubation at 18°C, and the microtubules remained intact (data not shown). To confirm that the dispersed vesicles we observed in the apical pole of nocodazole-treated cells originated from apical endosomes the following experiment was performed. IgA was internalized basolaterally and Fab fragments apically for 30 min at 37°C, the cells were nocodazole treated for 60 min at 4°C, and then reincubated for 60 min at 18°C with nocodazole. Both ligands were found colocalized in small vesicles across the apical region of the cell, confirming that the normal distribution of the apical endosomal compartment is dependent on microtubules (Fig. 11 *b*).

The above results suggest that microtubules are intimately involved in the organization of the apical tubulovesicular endosomal compartment. In fact the centralized distribution of this compartment may reflect the organization of this com-

partment around the centrosome. The centrosome is composed of a pair of centrioles and associated pericentriolar matter that forms the microtubule organizing center (MTOC). As described above, the apical IgA compartment is centrally distributed and radiates toward the margins of the cell. We have found that the MTOC of these cells, stained in Fig. 11 *c* with an antibody to γ tubulin (Stearns et al., 1991), was localized to the apex of the cell and in the center of the apical IgA compartment. In addition, we have found that IgA–HRP tubules/vesicles were frequently found concentrated around the centrosome (see Fig. 11 *d*; the centriole is marked with an "*") and confirm that the tubulovesicular apical compartment may be organized about this organelle.

Discussion

IgA Is Delivered from Basolateral to Apical Endosomes

In the currently accepted model for transcytosis, the pIgR and its ligand, IgA, are thought to be sorted in basolateral early endosomes into transcytotic vesicles that directly fuse with the apical plasma membrane. A major finding of our work is that transcytosis of the pIgR in MDCK cells works quite differently from this classic model. Our data suggests that IgA is delivered from basolateral to apical endosomes, and from there to the apical surface. When internalized from the basolateral surface of MDCK cells IgA is found to accumulate under the apical plasma membrane in a compartment that is accessible to two apically added membrane markers, anti-SC Fab fragments and avidin internalized from the biotinylated apical pole of the cell. Entry of IgA into the apical endosome occurred even in the presence of apical trypsin, which prevents internalization of IgA from the apical cell surface.

Our work is largely consistent with recent data from Barroso and Sztul (1994) in which they have analyzed the transcytotic pathway of $F(ab')_2$ fragments, derived from polyclonal antiserum to SC, in pIgR-expressing MDCK cells incubated at 17°C. Based on morphological data and release of labeled ligand from nocodazole-treated cells (similar to the analysis we have performed in Fig. 7), they have come to the same conclusion presented in this work that transcytosis involves transit from basolateral to apical endosomes.

The apical endosomal compartment we have described may be analogous to the recycling endosome compartment described in non polarized cells (see below). Much like these recycling endosomes in non-polarized cells, when the fluid-phase marker FITC–dextran was internalized apically it was largely excluded from the tubular aspects of the IgA-filled apical endosomal compartment. We believe that this reflects the tendency for fluid-phase markers to fill the more vesicular elements of the endosomal compartment and to be excluded from the membrane-rich, narrow tubules of this compartment (Rome, 1985; Mayor et al., 1993). Our data are consistent with the possibility that endocytosed fluid-phase markers and IgA may enter a common apical endosomal compartment but are then rapidly segregated by the mechanism described above. In fact, we can label the tubular aspects of the apical endosomal compartment when high concentrations of high specific activity HRP are internalized from the apical cell surface (Apodaca, G., and K. Mostov, unpublished observations). Similarly, Hollinshead and Tooze have described an elaborate anastamosing tubular endosomal compartment that is present in several different cell lines (Tooze and Hollinshead, 1991). This compartment is organized about the centrosome and contains recycling Tf. Most importantly, the fluid phase marker HRP is only detected in the tubular endosomal compartment when high-specific activity HRP is used at 10 mg/ml and internalized for at least 30 min at 37°C. This data is consistent with our observation that fluid-phase markers are largely excluded from, or at least not concentrated in, the tubular endosomal compartment to which IgA is delivered.

Previously it was thought that the early endosomes of MDCK cells were organized into distinct apical and basolateral systems with no inter-communication (Bomsel et al., 1989; Parton et al., 1989). This notion was based on the observation that fluid phase markers internalized from opposite poles of the cell only mixed in a common prelysosomal compartment. Moreover, apical and basolateral early endosomes labeled with fluid phase markers did not fuse in a cell-free assay (Bomsel et al., 1990). In contrast, our data and that of Barroso and Sztul (1994) and Hughson and Hopkins (1990) suggest that a pathway exists that allows for transport of membrane-bound proteins such as IgA and Tf between apical and basolateral endosomes. The exact mechanism by which IgA and Tf is delivered from the basolateral to apical endosomes is not known, although we do know that it requires microtubules. These ligands may be shuttled via vesicles that connect these two endosomal populations. Strictly speaking, however, these would not be "transcytotic vesicles" as they deliver proteins from basolateral to apical endosomes, and apparently they can deliver both IgA and Tf to the apical endosome. Alternatively, the apical and basolateral endosomes may be connected by tubules, similar to those seen for Tf in Hep-2 cells (Hopkins et al., 1990). Such tubules could be dynamic, transiently forming connections between the two endosomal compartments, and as such may be difficult to observe or preserve. In liver convoluted endosomal tubules are seen stretching virtually from one pole of the cell to the other (Geuze et al., 1984).

Transcytosis does not appear to be via the TGN, as little colocalization of IgA and TGN38 were observed. It should be noted, however, that IgA was occasionally observed in the Golgi stacks of hepatocytes (Geuze et al., 1984) and we have observed IgA–HRP occasionally associated with morphologically identifiable Golgi stacks. The question of Golgi involvement in transcytosis merits further investigation. In addition, it is unlikely that IgA transcytosis involves late endosomes for the following reasons: (*a*) there is little colocalization of IgA present in the apical endosomal compartment and the AC17 antigen, a lysosomal membrane protein; (*b*) the pathway for transcytosis overlaps considerably with the Tf-receptor, the classical marker for the early endosomal compartment; (*c*) the delivery of IgA from basolateral to apical endosomes occurs at 18°C, a temperature at which delivery to late endosomes is inhibited; and (*d*) entry of IgA into the apical endosomal compartment is apparent even after a 10-min internalization period at 37°C, a time frame too rapid to involve late endosomes. However, we cannot rule out that in transit from basolateral endosomes IgA very rapidly passes through a late endosomal compartment on its way to apical endosomes. This would not detract from

our conclusion that these apical recycling endosomes are a major station in the transcytotic pathway.

IgA-containing tubules/vesicles quite similar to those described in this work underly the bile canalicular surface in hepatocytes (Geuze et al., 1984; Hoppe et al., 1985; Barr and Hubbard, 1993); these were previously interpreted as unfused transcytotic tubules/vesicles. However, IgA can be endocytosed from the bile canalicular surface (Jones et al., 1984), so it has not been ruled out that the IgA in these tubules had not first reached this surface and been reinternalized. Two groups have reported isolation of vesicular fractions that are highly enriched in transcytosing IgA and pIgR (Quintart et al., 1989; Sztul et al., 1991). Given that the isolation procedures were based on enriching for the compartment containing the highest concentration of the receptor or ligand, and that IgA accumulates in the apical endosomal compartment, it is possible that these fractions are composed in part of this compartment. Consistent with this notion, the isolated fraction of IgA-containing vesicles described by Quintart et al. (1989) contained two markers that recycle at the bile canalicular surface.

It is not known if this compartment also contains fluid, endocytosed from the bile canlicular surface, as it is difficult to measure fluid phase endocytosis at this surface in liver. Of course further work will be necessary to determine if IgA is delivered from basolateral to apical endosomes in hepatocytes.

The mechanism by which IgA exits the apical endosome and fuses with the apical plasma membrane is unknown. The simplest possibility is that the IgA enters the same vesicles or tubules containing apically endocytosed proteins recycling back to the apical plasma membrane. However, we can not rule out the possibility that distinct carriers are used for recycling proteins and for transcytosing proteins that are passing through apical recycling endosomes enroute to the apical surface.

In a recent review Kelly speculated that transcytotic proteins may be delivered from basolateral to apical endosomes (Kelly, 1993). Our data provide direct support for this model. Kelly also proposed that a similar scheme may apply generally to other cell types, e.g., neurons, and that delivery of material from the "apical endosome" to the cell surface may be regulated by hormones and neurotransmitters, much like the release of synaptic vesicle occurs in neuronal cells. Consistent with this notion, we have found that release of both IgA or Tf from the apical recycling endosome compartment to the apical medium is stimulated by the action of protein kinase C (Cardone et al., 1994). The stimulation of transcytosis of both ligands is further evidence that both ligands may be in the same compartment.

Sorting in Apical Endosomes

Classically, sorting of basolaterally recycling membrane proteins away from transcytosing IgA was thought to occur in basolateral early endosomes (Geuze et al., 1984). However, our biochemical data suggests that a large fraction of both basolaterally endocytosed Tf and IgA are delivered to an apical endosomal compartment. We estimate that at 37°C approximately 50% of Tf resides in the compartment we have described. Moreover, in the DAB density-shift assay it was apparent that apically internalized pIgR ligand, Fab-HRP, was able to cross-link either basolaterally internalized IgA or Tf in apical recycling endosomes. In addition, we observe that IgA and Tf-receptor are morphologically colocalized in this compartment. Of course at the light level it is not possible to rule out that the IgA and Tf or the Tf-receptor are segregated into separate non-overlapping subdomains of the apical endosomal compartment.

We should note that Barroso and Sztul (1994) did not find that Tf colocalized with IgA at the apical pole of the cell. This may be due in part to their use of directly labeled Tf and $F(ab')_2$ ligand, which give a much lower level of signal than we have obtained in our analysis. Furthermore, their fixation protocol (10 min in 3% paraformaldehyde) was perhaps less stringent than the pH shift technique we have used in this study. The observations reported in our study confirm the work originally reported by Hughson and Hopkins (1990). Using EM they have found in Caco-2 cells that a substantial fraction of the apical Tf-containing structures were found to be accessible to apically endocytosed concanavalin A, suggesting that they are similar or identical to the apical endosomes that we have observed in MDCK cells.

In the present study it is not possible to determine if Tf is recycling predominantly from the peripheral basolateral endosomes or from apical recycling endosomes. In Caco-2 and A431 cells there appears to be a "short circuit" pathway for Tf recycling that is rapid ($t_{1/2}$ of 7.5 min) and an additional recycling pathway with slower kinetics. The "short circuit" pathway is thought to represent recycling from peripheral/basolateral endosomal structures. In A431 cells the slower recycling pathway is thought to involve a juxtanuclear, para-Golgi compartment, while in Caco-2 cells this recycling pathway is thought to involve the apical endosomal compartment described above.

One possible model is that in the basolateral endosome Tf and IgA would undergo one round of sorting into recycling and "transcytotic vesicles/tubules," respectively. Tf present in the latter structures would then reach the apical endosomal compartment and would have another chance to be sorted into the recycling pathway. This iterative model for sorting could account for the extraordinarily high accuracy of Tf recycling (99.8%) seen in some MDCK cell strains (Fuller and Simons, 1986). Such accuracy could be physiologically important, for instance in preventing loss of Tf and Fe (a limiting nutrient) into the urine by kidney cells. An alternative by not mutually exclusive model is that the apical endosomal compartment we have described is the polarized cell homologue of the para-Golgi Tf recycling endosome found in non-polarized cells. In both cases these compartments are tubular, organized about the centrosome, dispersed in nocodazole treated cells, and Tf is delivered to them from peripheral/basolateral endosomes (Hopkins, 1983; Mayor et al., 1993; McGraw et al., 1993). In this model the majority of Tf would be recycling through the apical endosomal compartment.

Role for Microtubules in the Organization of Apical Endosomes

Microtubules are known to be important in several steps of the endocytic pathway of MDCK cells including delivery of macromolecules from the early endosomes to late endosomes and delivery of basolateral ligands to the apical region

of the cell (Bomsel et al., 1990; Breitfeld et al., 1990; Hunziker et al., 1990). Here we report that the organization of apical early endosomes is dependent on microtubules. We find that the apical tubulovesicular elements containing IgA are concentrated around the centrosomes. In addition, if this compartment was preloaded with ligand and the cells then treated with nocodazole, the compartment fragmented and dispersed away from its centralized location in the apical region of the cell. Previous data indicated that recycling of apically endocytosed ligands bound to the pIgR was inhibited by nocodazole (Breitfeld et al., 1990; Hunziker et al., 1990), which also supports the notion that the function of apical endosomes is dependent on microtubules. It should be noted that although some microtubules appear to originate from the centrosomes of MDCK cells, many of the apically distributed microtubules may be organized at alternative sites near the apical plasma membrane (Bacallao et al., 1989). Finally, in the newly described hepatocyte cell line, WIF-B, the MTOC of these cells are found in numerous foci that abut the bile canalicular surface of the cell (Ihrke et al., 1994). Additional work will define if the organization of the tubular endosomes present under the bile canalicular surface of hepatocytes is dependent on microtubules.

A Model for Transcytosis and Polarized Sorting in MDCK Cells

In Fig. 12 we present a new model for transcytosis and polarized sorting in MDCK cells. IgA is internalized along with Tf and the fluid phase marker, FITC–dextran, into basolateral endosomes (step 1). The FITC–dextran is delivered in a microtubule dependent step to prelysosomes and ultimately to lysosomes. Although some of the Tf probably recycles from these basolateral endosomes, a large fraction of it is delivered along with the majority of IgA in a microtubule dependent step to an apical endosomal compartment (step 2). This compartment is a key site for polarized sorting and has several functions, including recycling of Tf to the basolateral cell surface, and recycling of apically internalized proteins to the apical plasma membrane. As such we refer to this compartment as the apical recycling endosome. This term emphasizes the function of this apical endosomal compartment and we suggest that it may be analogous to the para-Golgi recycling compartment described for Tf in nonpolarized cells. Although we show Tf and IgA in the same apical endosomal structures we can not rule out that the ligands enter separate microdomains of this compartment. IgA probably exits this compartment by entering vesicles/tubules containing proteins recycling back to the apical plasma membrane (step 3). However, we can rule out that there are separate mechanisms/pathways for delivery of transcytosing and recycling proteins to the apical cell surface. Ultimately, the pIgR is cleaved at the apical surface to SC releasing it and its ligand into secretions (step 4). Apically internalized fluid-phase markers are concentrated in the fluid-rich vesicular portions of the apical endosomal compartment and are transcytosed or delivered to prelysosomes. Although drawn in this manner, we do not know if these vesicular elements of the apical endosomal compartment are continuous with the tubular elements in which IgA accumulates.

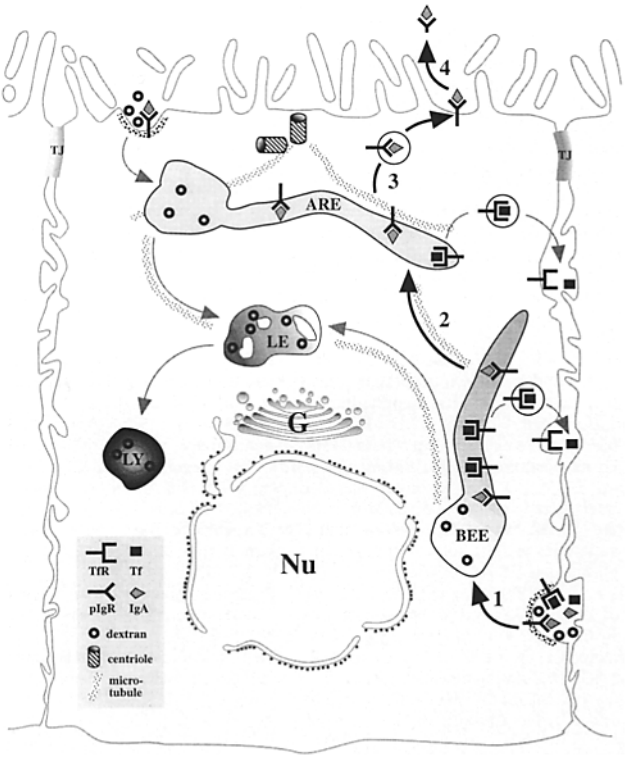


Figure 12. Model for transcytosis of the pIgR and polarized sorting in MDCK cells. See text for explanation. *ARE*, apical recycling endosome; *BEE*, basolateral early endosome; *G*, Golgi; *LE*, late endosome/prelysosome; *Ly*, lysosome; *Nu*, nucleus; *TJ*, tight junctions.

Our model for transcytosis simplifies the increasingly complex "road map" of membrane traffic pathways in polarized epithelial cells. The classic view postulated a minimum of three pathways for delivery of membrane proteins to the apical cell surface: direct delivery from the *trans*-Golgi network, recycling from apical early endosomes, and transcytosis from basolateral early endosomes (reviewed in Mostov et al., 1992). In our model it is not necessary to postulate a "private" transcytotic pathway from basolateral endosomes directly to the apical surface, along with specialized targeting machinery unique to this pathway. Instead, transcytosis is an integral part of the network of membrane traffic pathways, and parsimoniously utilizes pathways used by nontranscytosing molecules. Specifically IgA and Tf may be delivered to apical endosomes via the same tubules/vesicles. In this compartment Tf is recycled basolaterally, whereas IgA apparently switches to the same pathway used by apically recycling molecules to reach the apical plasma membrane.

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